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Prolonged incubation with low concentrations of mercury alters energy transfer and chlorophyll (Chl) *a* protein complexes in *Synechococcus* 6301: changes in Chl *a* absorption and emission characteristics and loss of the F695 emission band

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***Synechococcus* PCC 6301 cells grown in the presence of low sublethal levels of (about 2 μM) mercury induced alterations in chlorophyll (Chl) *a* absorption without significant alterations in phycocyanin. Chl *a* fluorescence emission in Hg^{2+}-raised cells showed a large (about 18 nm) blue shift in the peak emission. No major spectral changes in phycobilisome (PBsome) emission characteristic were noticed, indicating major structural alterations in Chl–protein complexes by incubation with Hg^{2+} ions. Low temperature (77 K) emission spectra of cells grown in the presence of Hg^{2+} showed a loss of the characteristic Chl *a* emission band at 695 nm (F695), which is known to be linked to photosystem II photochemistry and to originate from the Chl *a* of core antenna polypeptide CP 47 of photosystem II. The SDS–PAGE polypeptide profile of thylakoids indicates a loss of a polypeptide(s) with a molecular mass between 40 and 60 kDa by Hg^{2+} incubation of cells. Our results suggest that prolonged incubation of *Synechococcus* 6301 cells with low concentrations of Hg^{2+} affects the Chl *a* spectral properties and the structure of Chl–protein complexes.**

Keywords: chlorophyll *a*, energy transfer, fluorescence, mercury ions, photosystem, phycobilisomes, *Synechococcus* 6301

Introduction

Cyanobacteria are photosynthetic prokaryotes whose photochemical functions are identical to those of the higher plants (Bryant 1987); they both perform oxygenic photosynthesis. The prokaryotic nature of the cyanobacteria makes them an attractive system for studying the molecular organization of photosynthetic apparatus (Pakrasi & Vermaas 1992, Guikema & Sherman 1983). One of the major differences in the thylakoid membrane architecture of cyanobacteria lies in the arrangement of the light harvesting peripheral protein complex, the phycobilisome (PBsome) to the photosystem (PS) II, and PS I (Glazer 1982, Bullerjohn *et al.* 1987, Mandori & Melis 1987). The PS II core complex of cyanobacteria comprises D1 and D2 polypeptides, Cyb 559, 47 and 43 kDa chlorophyll (Chl) protein complexes (Barber *et al.* 1987, Nanba & Satoh 1987, Vermaas & Ikeuchi 1991), a cytochrome, cyt 550, a 33 kDa polypeptide and other low molecular weight polypeptides (Shen & Inoue 1993) that are required for efficient PS II catalyzed O_2 evolution.

Cyanobacteria exhibit characteristic fluorescence emissions both at room and at liquid nitrogen (77 K) temperatures (Cho & Govindjee 1970, Papageorgiou 1975, Fork & Mohanty 1986). The excitation of cyanobacterial cells with light primarily absorbed by phycocyanin (PC) and allophycocyanin (APC) of PBsomes produces emission from PBsomes and from Chl *a* of PS II. At room temperature, the emission from PS I is negligible (Goedheer 1968). At 77 K, the PBsome excitation evokes four peak emissions: F665, F685, F625 and F720. At 77 K, light mainly absorbed by Chl *a*, the fluorescence emission spectrum exhibits F686, F695 and F720 bands. Of these, F685 and F696 originate from PS II, F665 from PBsome, and F720 from PS I (Fork & Mohanty 1986). Krey & Govindjee (1966) suggested that a F695 band in the red alga *Prophyridium cruentum* is

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intimately linked to PS II photochemistry. Breton (1982) suggested this 77 K emission band is linked to the acceptor side of PS II. A variety of treatments that affect PS II organization and photochemistry quench the F696 emission (Mohanty *et al.* 1971, 1985, Singhal *et al.* 1981, Blubaugh & Govindjee 1988, Babu *et al.* 1991). F695 has been previously identified with the Chl *a*–protein complex, CP47, of PS II (Nakatani *et al.* 1984, Webb & Punnet 1989). Thus both structural and functional alterations at PS II affect F696 emission.

A large number of toxic metal ions affect electron transport and energy transfer in cyanobacteria (Murthy & Mohanty 1991a). Mercury (Hg^{2+}) ions not only inhibit electron transport at multiple sites (Murthy *et al.* 1990, Murthy & Mohanty 1993a, b) but also alter energy transfer from PC to Chl *a* of PS II in *Spirulina platensis* (Murthy & Mohanty 1991a, b, 1993a). Unlike *Spirulina* cells, the cells of unicellular alga *Synechococcus* 6301 (*Anacystis nidulans*) do not exhibit any major inhibitory effects upon short-term (about 5 min) exposure to Hg^{2+} ions. We thus studied the effect of Hg^{2+} ions in *Synechococcus* 6301 cells by incubating the cells in the presence of low, sublethal levels (1–5 μM) of $HgCl_2$ for long (48 h) periods. In this paper, we show that such long-term exposure of cells to low levels of Hg^{2+} ions induces drastic spectral changes in Chl *a*, thereby affecting energy transfer processes and causing loss of the F695 emission band, which suggests structural and functional alterations at PS II.

Materials and methods

Synechococcus PCC 6301 was grown axenically in BG-11 medium (Stanier *et al.* 1981, Murthy & Mohanty 1991b) at 25 ± 2°C under continuous illumination (about 20 W m^{-2}). Throughout the growth period, the culture was agitated by filtered air. Cells at the logarithmic phase were harvested by centrifugation at 10 000 *g* for 5 min, washed once with fresh growth medium and finally suspended in the same medium at 5 μg Chl *a* ml^{-1} of suspension. After $HgCl_2$ addition, the mercury-treated and control cultures were allowed to grow in light for 48 h. Thylakoid membranes from Hg^{2+}-incubated and control cells were prepared by following the procedure of Katoh (1988). Photochemical activities were assayed polarographically with a Clark-type oxygen electrode (Hansatek, Norfolk, UK) at 25°C under saturating light intensity (about 430 W m^{-2}). The 3 ml reaction mixture used for the assay of PS II catalyzed electron transport contained a HEPES suspension buffer (25 mM HEPES NaOH, pH 7.5), 0.5 μM parabenzoquinone (PBQ) and cells equivalent to 15 μg Chl.

Absorption spectra of suspensions of intact cells were recorded using a Hitachi 557 double beam spectrophotometer. Fluorescence emitted by excitation at 440 nm was recorded with a Perkin Elmer LS-5 spectrofluorometer. Cells equivalent to 5 μg Chl ml^{-1} were used for all fluorometric assays. Spectra have not been corrected for the spectral response of the fluorometer. Polypeptide patterns of thylakoids were analyzed by SDS–PAGE according to the method of Laemmli (1970). The concentration of Chl was determined by the method of Mackinney (1941).

Results and discussion

Cells of *Synechococcus* 6301 incubated with low concentrations (1–2.5 μM) of $HgCl_2$ for 48 h showed a decrease of the Chl *a* content. In 2 μM $HgCl_2$ grown cells, the Chl *a* content decreased by 50% (see Table 1). The decrease in pigment content was time dependent. After 48 h of incubation, a 50% drop in Chl *a* content with 2 μM concentration of mercury was observed. The loss in PC by Hg^{2+} ions during the same period was marginal compared with Chl *a* (data not shown). Unlike *Spirulina* (Murthy & Mohanty 1991a, b), the Hg^{2+}-induced changes in *Synechococcus* PCC 6301 are primarily in the thylakoid membranes rather than the PBsomes.

The presence of Hg^{2+} ions caused a decrease in both the red and the Soret band of Chl *a* absorption, and had almost no or marginal effect on PC absorption (Figure 1A). The absorption in the carotenoid region also decreased. In addition, mercury caused a blue shift (2 nm) in the Chl *a* peak absorption. No change in the peak position due to PC absorption was noted in the spectrum (Figure 1A).

The measurement of low temperature absorption spectra of (2 μM) Hg^{2+}-incubated cells indicated that carotenoid absorbance at 460 nm was more suppressed than at 496 nm (Figure 1B). We also note that the 418:435 nm peak ratio of Hg^{2+}-treated cells increased, suggesting alterations in pigment protein interactions. Since at room temperature most of the fluorescence comes from PS II (Goedheer 1968), we measured the emission spectrum of mercury-incubated cells to characterize the nature of alterations in PS II organization. The control cells excited at 440 nm exhibited an emission peak at 683 nm, originating from Chl *a*, and a shoulder at about 650 nm, due to PC and APC (Figure 2). These spectral characteristics are in agreement with the results of Singhal *et al.* (1981) for *A. nidulans*. Incubation of cells with low (2 μM) concentrations of mercury for 48 h caused a drastic shift in the peak position (about 18 nm) towards the blue region of the spectrum with a small increase

Table 1. Effect of different concentrations of mercury on Chl *a* content of the cyanobacterium *Synechococcus* PCC 6301 after 48 h of incubation

$HgCl_2$ (μM)	[Chlorophyll] (μg ml^{-1})
0	4.0 ± 0.3
1	4.1 ± 0.2
1.5	2.5 ± 0.3
2.0	1.9 ± 0.1
2.5	1.0 ± 0.08

The values are averages of three separate experiments ± SD ($n = 4$). Cells equivalent to about 1×10^8 cells were present per 1 ml of suspension in each case.

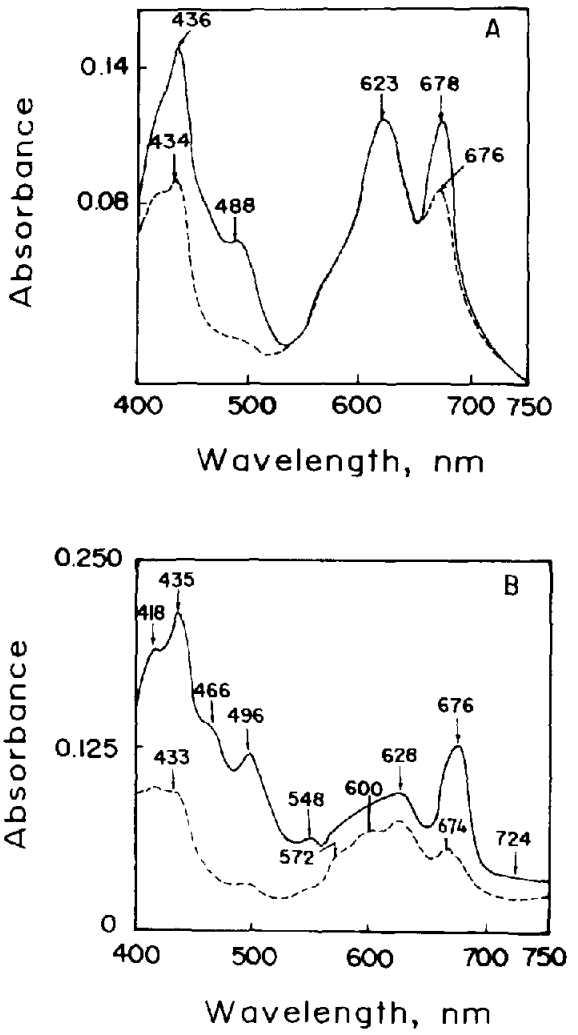

Figure 1. Effect of Hg^{2+} (2 μM) on room temperature (**A**) and low temperature (77 K) (**B**) absorption spectra of intact *Synechococcus* cells. Cells were suspended in 70% glycerol for low temperature spectral measurements. ——, Control; – – –, 2 μM $HgCl_2$.

in the fluorescence emission intensity (Figure 2). This apparent shift in the emission characteristic reflects the interruption of energy transfer from APC to Chl *a*. This causes the emission at 665 nm, due to APC, to rise as a peak and Chl *a* emission to decrease (Figure 2).

To further investigate the Hg^{2+}-induced changes, we measured the excitation spectra of control and Hg^{2+}-grown cells (Figure 3). In the control, the F685 nm emission showed a broad band with excitation peaks or shoulders at 667, 650 and 630 nm, reflecting the relative contributions of these chromophores of PBsomes to emission at 685 nm. The excitation band at 667 nm is due to APC and the excitation bands at 630 and 650 nm are due to PC and APC (Fork & Mohanty 1986). In Hg^{2+}-grown cells we noted that the increase in the intensity is greater at 667 than at 630 nm. The enhanced excitation intensity for F685 emission in these Hg^{2+}-grown cells could be due to the fact that besides Chl *a*, the 683–685 nm emission (F685) has also a substantial contribution from the APC β chromophore which transfers energy to Chl *a* (Mohanty *et al.* 191985). These results, thus, suggest that prolonged incubation with Hg^{2+} ions obstructs energy transfer from APC to Chl *a*.

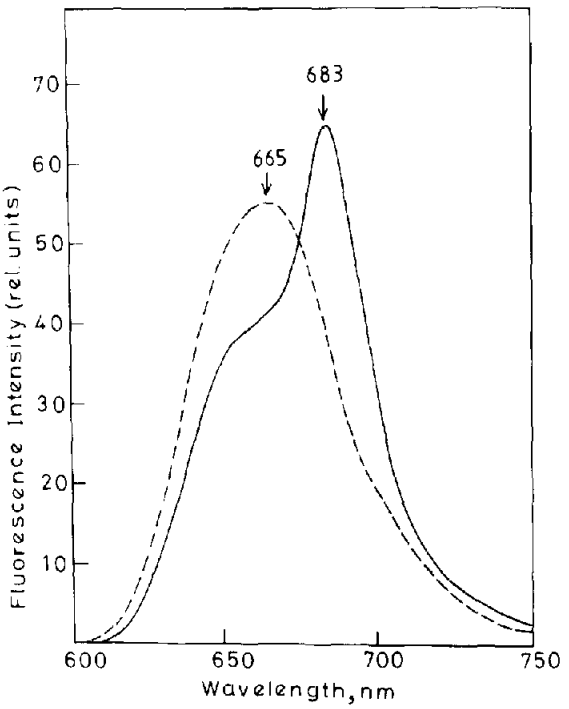

Figure 2. Room temperature fluorescence emission spectrum of control and Hg^{2+}-treated (mercury raised) cells of *Synechococcus*. Cells were excited with 440 nm light. Slit widths for both excitation and emission were 5 nm. Cells equivalent to 5 μg Chl were used for spectral measurements. ——, Control; – – –, 2 μM $HgCl_2$.

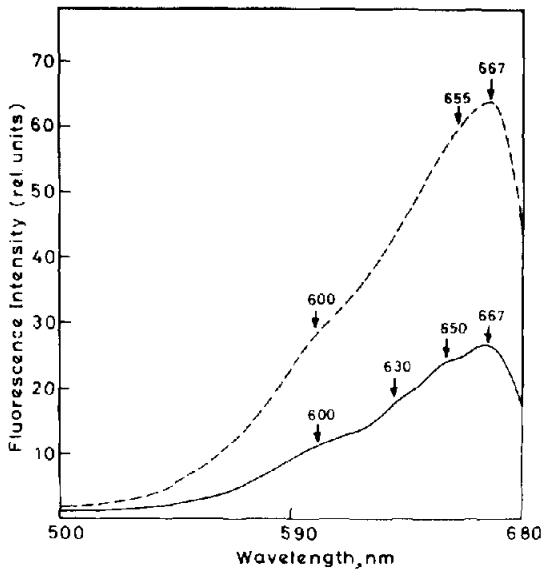

Figure 3. Excitation spectra of control and Hg^{2+} (2 μM)-incubated intact cells of *Synechococcus*. Excitation spectra were measured at F685 from 500 to ~680 nm. Slit widths for both excitation and emission were 5 nm. Cells equivalent to 5 μg Chl were used for spectral measurements. ——, Control; – – , 2 μM $HgCl_2$.

To examine further the low temperature (77 K) emission, spectra were recorded in control and in Hg^{2+}-grown cells, by exciting with a broad band blue light which is mostly absorbed by Chl *a*. Four emission peaks were observed in control spectrum, as shown in Figure 4. The emission at 650 nm is due to PC, 685 nm is mostly due to APC β and some Chl *a* (Mohanty *et al.* 1985), 695 nm from Chl *a* of PS II core antenna (CP47) and 715 nm is due to Chl *a* of PS I. This control spectrum is similar to the spectrum of *A. nidulans* shown earlier by Singhal *et al.* (1981). In Hg^{2+}-raised cells, besides marginal changes in fluorescence emission intensity, small blue shifts both at 685 and 715 nm

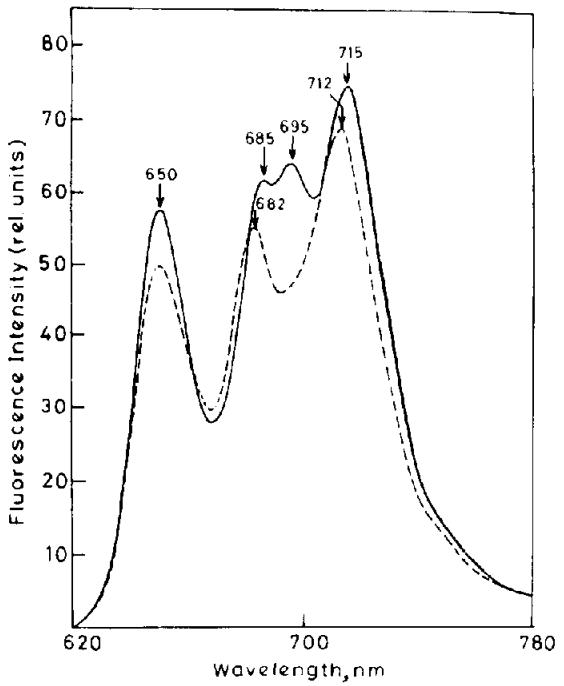

Figure 4. Low temperature (77 K) fluorescence emission spectra of *Synechococcus* cells. Frozen cells were excited with 440 nm light. Slit widths for both excitation and emission were 5 nm. Cells equivalent to 2 μg Chl were used for spectral measurements. ——, Control; – – –, 2 μM $HgCl_2$.

Table 2. Mercury induced alterations in PS II catalyzed electron transport and Chl *a* fluorescence in *Synechococcus* PCC 6803 cells

Sample	Percent activity PS II catalyzed electron transport ($H_2O \rightarrow pBQ$)	Chl *a* fluorescence +DCMU/−DCMU
Control	100	2.0
$HgCl_2$ (2 μM)	20	1.1

$HgCl_2$ incubation was for 48 h. Other details were given in Materials and methods. Control electron transport activity (100%) was equal to 400 μmol O_2 evolved mg Chl^{-1} h^{-1}. Steady state Chl *a* fluorescence was measured at 686 nm with 440 nm excitation; 5 nm slit width with equivalent amounts of Chl *a* (5 μg ml^{-1}) in both cases. DCMU, 10 μM.

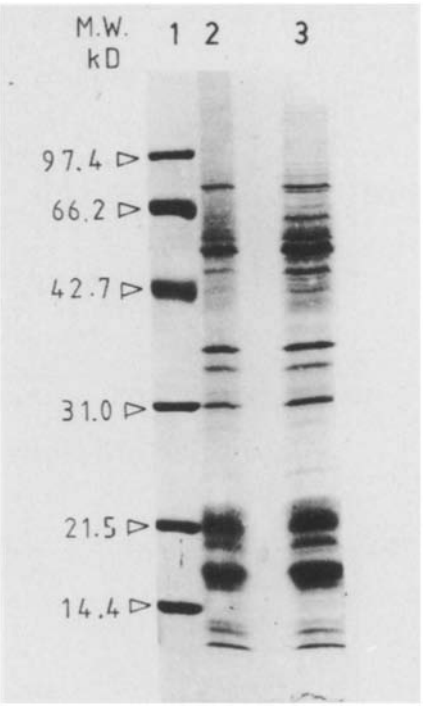

Figure 5. SDS–PAGE electrophoretic profile of thylakoid membranes. Lane 1 shows the polypeptide pattern of Bio-Rad markers. Lane 2 shows the polypeptide profile of 2 μM mercury ($HgCl_2$)-raised cell thylakoids. Lane 3 shows the polypeptide composition of control (untreated) thylakoids. Thylakoids equivalent to 15 μg protein were used for electrophoretic studies.

occurred. However, no shift in the position of the peak due to PC was noted. Also, the 685 nm emission band was slightly affected by Hg^{2+} ions, but the 695 nm emission band was totally suppressed by the presence of mercury. The F685 emission is contributed to by both Chl *a* and APC β of PS II, while the F695 band, as discussed earlier, arises from core antenna CP 47 of PS II (Anbudurai & Pakrasi 1993, Nakatani *et al.* 1984). This PS I emission band was also blue shifted by 3 nm (Figure 4). The blue shift in the peak positions reflects the drastic alterations in the structure of the pigment proteins of thylakoid membranes (Singhal *et al.* 1984). The loss of the 695 nm peak in the 77 K spectrum is a good indicator of alterations of PS II photochemistry, as shown by Blubaugh & Govindjee (1989) by the use of the inhibitor disulfirm (an iron chelator), which inhibits PS II electron transport at the acceptor side. Our results also show that the presence of Hg^{2+} reduces O_2 evolution (Table 2). Thus, the loss of O_2 evolution and the loss of the F696 band suggest that Hg^{2+} ions possibly affect the PS II core complex.

Webb & Punnet (1989) have shown that the mutant *Synechococcus* strain PCC 7002, which is defective in the accumulation of CP 47, shows a loss in the F696 band in the 77 K emission spectrum, which suggests that the loss of CP 47 causes the loss of 695 emission. To study alterations, if any, in the pattern of the thylakoid proteins, we analyzed the SDS PAGE profiles of control and Hg^{2+}-

raised *Synechococcus* thylakoid membranes. The molecular compositions of total thylakoid proteins ranged from 17.5 to 94 kDa on the SDS–PAGE profile (Figure 5). The 18.5 and 20.5 kDa polypeptides correspond to the α and β subunits of phycobiliproteins, and the rest of the polypeptides are colorless polypeptides of PBsomes and Chl *a* containing proteins. From the PAGE profile it can be seen that the Hg^{2+}-induced a decrease in polypeptides in the range of 40–60 kDa. This loss appears to be of a similar nature to that reported by Webb & Punnet (1989) in their CP 47 defective mutant. A recent study of Anbudurai & Pakrasi suggests that loss in F696 emission does not necessarily reflect the loss of the CP 47 apoprotein. However, our results indicate that upon prolonged incubation of cells of *Synechococcus* with low concentrations of Hg^{2+}, the Hg^{2+} ions not only alter energy transfer from PBsome to Chl *a* but also induce structural changes in the core pigment proten compositions of thylakoid proteins.

Our result showing the effect of incubation of intact cells of *Synechococcus* with low levels of Hg^{2+} ions on Chl *a* fluorescence characteristics is new and interesting as mercury induced drastic changes in the absorption and emission of Chl *a* of PS II, but had no effect on PBsome spectral characteristics. The reason for this type of differential effect may be due to the fact that this metal ion affects the photosynthetic electron transport system (Trebst 1974) at multiple sites in a concentration- and time-dependent manner (Murthy *et al.* 1990, Murthy & Mohanty 1993b). The sensitivity of Hg^{2+} to inhibit PS II photochemistry is well known and such an inhibition of PS II activity is also, as in the case of Ca^{2+} depletion (Brand *et al.* 1983, Mohanty *et al.* 1985), known to specifically induce changes in Chl *a* emission and loss of the 77 K F695 emission band in cyanobacteria but not in PBsomes. It is possible that the presence of Hg^{2+} induces similar effects by arresting PS II photochemistry in *Synechococcus*. However, the exact cause for such differential effects of Hg^{2+} on proteins of PBsome and PS II awaits further investigation.

In summary, we wish to conclude that the prolonged incubation of sublethal levels of Hg^{2+} with *Synechococcus* 6301 (i) causes interruption of energy transfer from PBsomes to Chl *a*, (ii) induces drastic changes in absorption and emission features of the treated *Synechococcus* 6301 cells, and (iii) brings about structural changes in thylakoid protein, possibly linked to the PS II core complex, thereby imposing a loss in the F696 emission band at 77 K.

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