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Characterization of the alterations in the thylakoid pigment proteins under cold stress in the intact cells of the cyanobacterium; *spirulina platensis*

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Abstract

The response of *Spirulina platensis* to cold stress (10°C) for 12h was investigated by incubating the intact cells in light of moderate intensity. It inhibits energy transfer from phycocyanin to chlorophyll *a* and specifically affecting the anchor and linker polypeptides, in cyanobacterium. Cold treatment of intact cells also resulted in the alteration of other thylakoid membrane proteins: most prominently, a dramatic diminishment of 37.2-kDa and 97.1 kDa proteins, and appearance of a new polypeptide around 47kDa were observed in SDS-PAGE. The SDS Gel of PBsomes isolated from cold stressed samples exhibited the loss in the content of linker polypeptides in the range of 32-37kDa particularly degradation of 35kDa which links PC to APC. Therefore we conclude that cold stress for long term has various effects on photosynthetic electron transport activities due to marked alterations in the composition of thylakoid membrane proteins.

INTRODUCTION

Each plant species has an optimum temperature for growth and development [1] .More than 70% of the earth exists as cold ecosystems that has a stable temperature below or close to the freezing point of water and hence cold stress is a major abiotic stress problem to plant productivity and growth adaptations [2]. Among the cell structure, the chloroplast is usually the only organelle that is more rapidly and deeply affected during cold stress [3-4]. The bulk of the Chl and carotenoid present within the chloroplast thylakoid membrane is bound to be LHC b. and LHC a families of light harvesting polypeptides, associated with PS II and PS I, respectively [5]. Winter conifers long term changes in the organization of the photosynthetic apparatus that induces a decrease in the number of functional PS II reaction centers, a loss of light harvesting Chl and the formation of a large thykaloid protein complex involved in LHC-II, PS II, PS I [6-7]

The major site of photo inhibition in PS II is the inactivation of D1 protein [8-9]. This inactivated protein must be newly synthesized to restore PS II activity. The actual extent of PS II photoinhibition in vivo depends on the balance between the inactivation of D1 and the recovery process which involves insertion of new D1 molecule into the thylakoid and their incorporation into the PS I complex. Recovery from PS II photoinhibition is strongly temperature dependant i.e. low temperature will decrease the rate of repair [10]. Photo damage becomes apparent as low temperature interferes with the normal replacement rate of D1 in the turn over repair cycle. This has been attributed to changes in the expression of psb A, the plastid gene that encodes D1 and directs temperature effect on membranes [11].

The effects of the growth under chilling condition on PS I, PS II, CO_2 assimilation have been investigated [12]. Therefore a limited number of reports showed that PS I has a greater chilling sensitivity than PS II. As for other plant species, such limitations could be attributed to degradation of pigment complexes and loss of photochemical efficiency, increase of damage and retardation of repair processes at the PS I and PS II level (e.g. in D_1 protein), restriction of electron transport, enzyme activities of carbohydrate metabolism and increase of chloroplast membrane permeability [13-14] Several studies demonstrated that thylakoid membrane proteins were affected by many environmental factors but the reports on cold stress alterations in the thylakoid membranes proteins in cyanobacterium: *spirulina platensis* is scanty so we have made an attempt in this paper to demonstrate the effects on the thylakoid proteins under cold stress.

MATERIALS AND METHODS:

Plant material and cold stress treatment

Spirulina platensis cells were grown at 25±2°C in Zarrouk's medium [15] under continuous illumination (20 Wm^{-2}). The culture was bubbled with filtered air. Cells from the late log grown cultures were harvested by centrifuging at 6,000 X g for 10 min. The collected cells were suspended in 25mM HEPES-NaOH buffer (pH 7.5) at a Chl conc of 200 µg mL-1. Samples were incubated at low temperature (10-25°C) for short term (15-60 min) where as for long term the thylakoid were exposed to 12 h in conical flasks under constant stirring. The spectral measurements were taken immediately after giving the treatment.

Isolation of phycobilisomes

The PBSs were isolated from *Spirulina* cells according to the method of [16] with some modifications. After harvesting, *Spirulina* cells were washed twice with 1M potassium phosphate (K-PO4) buffer at pH 7.0. The cells were resuspended in 10 ml of 1M K-PO4 buffer at pH 7.0 containing 1 mM PMSF, 1 mM sodium azide, 2 mM EDTA. The PBSs have been isolated by treating with triton X-100 and layering the resulted supernatant on sucrose density gradient by following the procedure of [17]. The PBSs were recovered from the 1.0M region as an intense blue band. Sucrose was removed from the PBSs by passing them through a sephadex G-25 column, equilibrated with 1M phosphate buffer pH 7.0. After removing sucrose, these PBSs were used for spectral measurements.

Absorption and room temperature PC fluorescence emission spectra

Absorption spectra were recorded on Jasco UV- Vis spectrophotometer. The cells were suspended in the reaction buffer (25 mM HEPES-NaOH, pH 7.5) at the concentration of 6

µg of Chl a per ml. In the case of PBSs, they were suspended in the 0.75 KPO4 buffer pH 7.0 at the concentration of 30 mg protein per ml. This cell suspension was taken for scanning the absorption spectra from 400 nm to 750 nm in the visible region. All these absorption spectra were taken at the room temperature and they were not corrected for spectral sensitivity. Hitachi spectrofluorimeter was used to record fluorescence emission and excitation spectra. Sample preparation was done in the same way as described above. The reaction mixture contained reaction buffer and the intact cells equivalent to 5 µg Chl *a* concentration. 5 nm slit width were maintained for recording both the excitation and emission spectra. The intact cells or PBSs or pigment proteins were excited at 545 nm to excite PC specifically since these organisms contain PXB chromophore which absorbs light at 580 nm [18].

SDS-PAGE analysis.

Total soluble proteins of PBS samples were analyzed on SDS-PAGE according to the method of [19]. The stacking gel was 4% polyacrylamide and the running gel was 12.5% polyacrylamide used for the separation of polypeptides. Samples containing 1 mg/ml protein was solubilised by boiling for 2 min in sample buffer containing 5% glycerol 0.1M Tris-HCl, (pH 6.8), 2% SDS and 100 µM mercaptoethanol along with 0.1% of bromophenol blue. Thylakoid proteins were loaded on equal Chl basis, 12µg per lane.The electrophoresis was conducted by adopting the procedure of Murthy (1991)[17]. The molecular mass of the resolved proteins was calculated by using Bio-Rad markers (phosphorylase B 97,400; Bovine albumin 66,200; Ovalbumin 42,700; Carbonic anhydrase 31,000; Trypsin inhibitor 21,500; Lysozyme 14,400 daltons).

RESULTS AND DISCUSSION:

From our previous paper it is clear that PS II catalysed electron transport activity altered due to alterations in the light harvesting complex which is responsible for the altered photochemistry under cold stress. Phycobilisomes are the major light harvesting pigments proteins complex in PS II of cyanobacteria. Therefore to exactly pinpoint the target for cold stress both absorption and Fluorescence measurements of phycobilisomes have been made using intact cells of *Spirulina* (Fig 1, 2, 3). Fig 1 shows the absorption characteristics of control cells. The peak at 679 nm is due to the absorption of Chl *a* ; peak at 630 nm is due to the absorption of PC of PBsomes; a hump at 484 nm is due to the absorption of carotenoids; and a peak at 437 nm is due to soret band of Chl *a [20]*. After low temperature treatment (10°C) there was a drastic decrease in the absorption at 630 nm with a 2 nm red shift. There was no change in absorption capacities of Chl *a* and carotenoid and xanthophyll. Since room temperature PC florescence emission of *Spirulina* was made (Fig 2). In control cells, excited at 545 nm, an emission peak at 647 nm emanating from PC was prominent in the spectrum [21,20]. Cold stress (10°C) caused decrease in the fluorescent intensity and induced 5 nm blue shift. The decrease in the fluorescence intensity indicates the uncoupling of energy transfer and blue shift shows the structural alterations in the pigment proteins of phycocyanin. similar reports were made under the influence of nitrogen stress and mercury stress in the same cyanobacteria [17,22]. To examine the specific target of cold stress in PS II, the Chl fluorescence emission spectra at low temperature (77K) was measured by exciting with 440 nm light beam. In control, three prominent peaks were observed as shown in Fig 3; emission band 645 nm is due to PC; 695 nm band is due to PS II reaction center

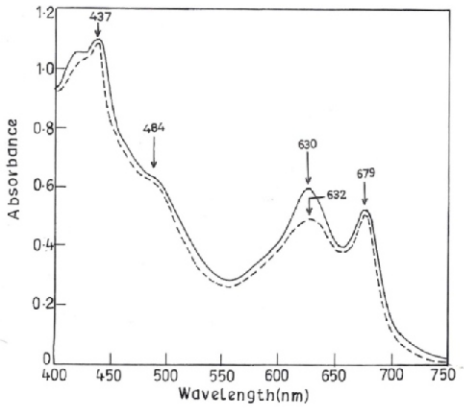

Fig 1: Effect of cold stress on the absorption spectra of the intact cells of the cyanobacterium, *Spirulina platensis*. Cells were treated at 10°C for 45 min before measurements were made.

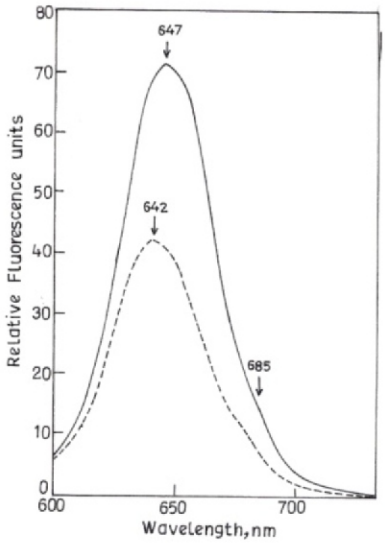

Fig 2: Effect of cold stress (10°C) on PC fluorescence emission spectra of intact cells of *Spirulina.*

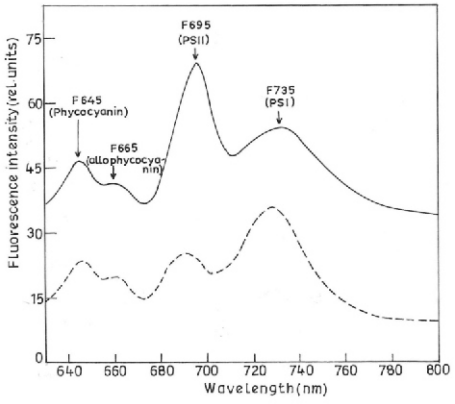

Fig 3: Effect of cold stress (10°C) on 77 K fluorescence emission spectra of intact cells of *Spirulina.* Cells were excited with 440nm.

Chl ; 735 nm band is due to PS I reaction center Chl and the hump at 665 nm is due to APC. Low temperature treatment (10°C) mainly caused the suppression of the 695 nm peak with 3 nm blue shift. In addition the ratio of fluorescence intensity between control and low temperature treated samples (F695/F735) decreased indicating the change in the spillover of energy between the two photosystems. Similar reports were made in *Synechococcus* 6301 under the influence of sublethal concentrations of mercury (2 µ MOLAR) by Murthy *et al.*(1995)[23].

After studying the functional aspects of pigment proteins an attempt has been made to determine the affect of cold stress on polypeptide profile of *Spirulina* cell total protein by using SDS PAGE analysis. To achieve this, cells were incubated at 10°C for 12 h and then the polypeptide analysis has been made. SDS-PAGE analysis of total protein of untreated *Spirulina* resolved its components in the molecular range of 97 to 13.5 kDa (Fig 4). The phycobiliproteins of intact cells were in the range of 16 to 22 kDa. These proteins are chromophore linked and they can be seen on the gel prior to staining with Coomassie Blue R-250. Fig 4 shows the polypeptide profile of *Spirulina* control and cold stress treated (10°C) cells for 12h. The low temperature caused the decrease mainly in 37.2 kDa and 97.1 kDa polypeptides. There is no change in the chromophore bearing polpeptide which are in the range from 16-22 kDa. These two polypeptides (97.1 and 37.2 kDa) according to literature are related to anchor and linker polypeptides respectively. In addition it also caused the appearance of a new polypeptide around 47 kDa . This polypeptide could be cold stress response protein which helps in the minimization of cold induced damage. The above studies were made *Spirulina* cells under *in vivo* conditions. Always there is need for the comparison of *in vitro* result with *in vivo* studies to have a clear understanding about the problem. Hence further studies were mainly concentrated under *in vitro* conditions. After studying the effect of cold stress on intact cells it is clear that PBsomes are the main target for cold stress.

Therefore an attempt has been made to isolate PBsomes form control as well as low temperature treated *Spirulina* cells. Fig 5 shows the presence of PBsomes in the sucrose density gradient centrifugation. A thick blue colour band was observed at 1 M region of sucrose after ultracentrifugation. In cold stress treated samples exhibited a thick blue layer at 1 M region in addition to another light blue band at 0.5 M sucrose gradient. This clearly demonstrated that cold stress caused the dissociation of some of the pigment proteins from intact PBsomes. To determine the functional integrity of PBsomes, spectral properties of phycobilisomes were determined. Fig 6 shows the spectral characteristics of the isolated PBsomes. The absorption spectrum of PBsomes exhibited main peak at 613 nm due to PC and a pronounced shoulder at 650 nm due to APC as shown in Fig 6. The PBsomes isolated from cold stressed samples caused decrease in the absorption and shifted the peak towards blue region of the spectrum by 3 nm indicating the structural alterations in PBsomes. Since fluorescence and energy transfer is related to absorption and fluorescence emission, spectra of PBsomes was measured by exciting the PBsomes with 545 nm light beam showed the characteristics emission peak at 669 nm emanating from the longer wavelength absorbing species (Fig 7). This indicates that the energy transfer in the isolated PBsomes is intact and it is getting transferred from PC→APC. These spectral characteristics are in agreement with the observation of [24]. The cold stress (10°C) for 12h caused the decrease in the fluorescence

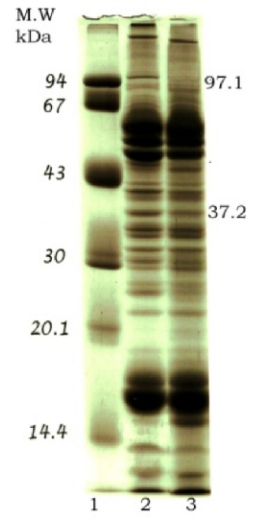

Fig 4: SDS-PAGE polypeptide profile of total soluble thylakoid proteins of control *Spirulina* cells and treated (10°C) for 12 h.

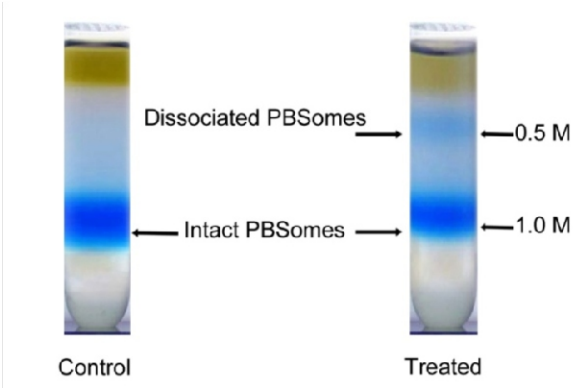

Fig 5: Sucrose density gradient sedimentation profile of intact PBsomes isolated from Control and Treated (12h cold stressed) samples of *Spirulina platensis* cells.

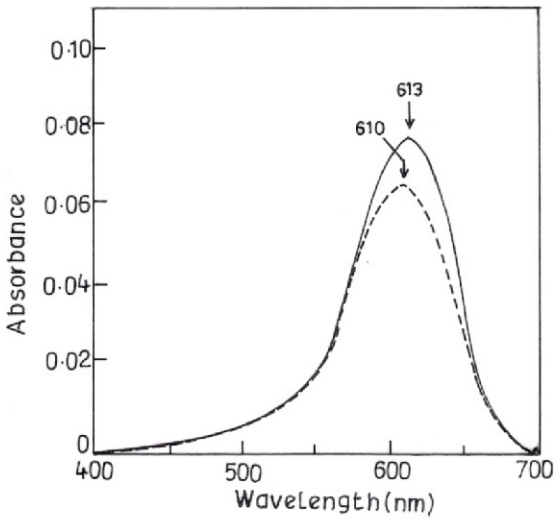

Fig 6: Absorption spectrum of isolated PBsomes from Control and Treated *Spirulina* cells.

emission and shifted the peak by 4 nm towards the blue region indicating the uncoupling of energy transfer from PC→APC B. This loss clearly demonstrated the uncoupling of energy from PC to APC B in isolated PBsomes. Similar reports were early made by [25] under UV-B. The anchor polypeptide has been demonstrated to be very susceptible for proteolytic degradation and hence utmost care is necessary for preventing the degradation during the isolation procedures [24].

The PBsomes isolated from cold stressed samples exhibited the loss in the content of linker polypeptides in the range of 32-37 kDa particularly low temperature induced the degradation of 35 kDa which links PC to APC. These results are in agreement with the observations of Sah *et al*. (1998) [26]. From the analysis of spectral measurement, it is clear that phycobiliproteins are the major targets for cold stress. Hence, to identify the structural organization, the PBsomes have been isolated from 12 h cold stress (10°C) cells. The electrophoretogram showed the polypeptide profile of control and cold stress PBsomes of *Spirulina* (Fig 8). Besides the bilin-carrying subunits of phycobiliproteins, five non-pigmented polypeptides were clearly seen (lane 2), associated with PBsomes. The polypeptide at 94 kDa in the electrophoretogram is the anchor polypeptide which links the PBsomes to the thylakoid membrane. The polypeptides between 32 and 37 kDa are linkers which help in the attachment of PC-PC rods (Yamanaka and Glazer, 1980). The intense bands, 16 to 22 kDa are the subunits of phycobiliproteins (both PC and APC). The presence of single band of the anchor polypeptide indicated after long term incubation (12 h). Similar loss of linker polypeptide was early reported in synecoccous 6301 under nitrogen stress by [27]. Thus alterations in the linker polypeptide were responsible for the change in the energy transfer from PC APCB in PBsomes isolated from *Spirulina plantensis* under cold stress.

CONCLUSION

The studies were made by incubating cells at low temperature (10°C) for short period (45 min) and long duration (12 h). Cold stress causes alterations in the energy transfer of phycobilisomes under *in vivo* and *in vitro* conditions as evident from spectral alterations in absorption as well as fluorescence emission. Low temperatures mainly affect the linker polypeptide which attaches PC-PC and the molecular weight of this polypeptide is approximately 35 kDa. During short term incubation cold stress causes the alterations in chromophore protein interaction of PC as revealed from the CD spectrum as shift in the peak from 350 nm to 360 nm.

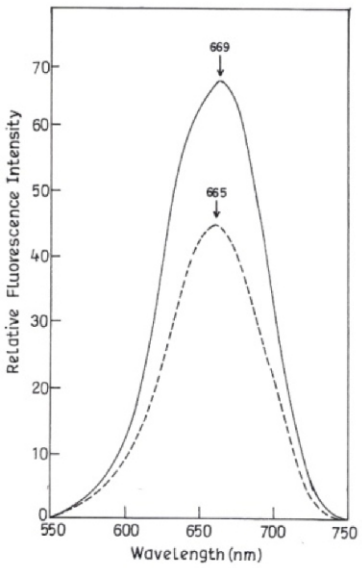

Fig 7: Fluorescence emission spectrum of isolated PBsomes from control and treated *Spirulina* cells.

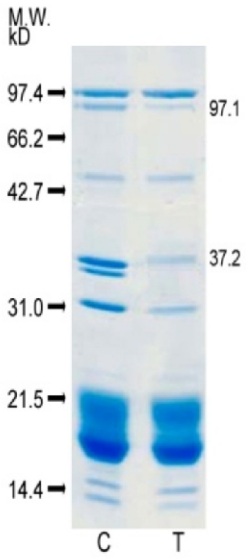

Fig 7: Fluorescence emission spectrum of isolated PBsomes from control and treated *Spirulina* cells.

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