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The Effects of Salt Stress on Photosynthetic Electron Transport and Thylakoid Membrane Proteins in the Cyanobacterium *Spirulina platensis*

Putty-Reddy Sudhir[†,‡], Denys Pogoryelov[‡], László Kovács[‡] and Gyõzõ Garab[‡] and Sistla D.S. Murthy[†,*]

[†]Department of Biochemistry, Sri Venkateswara University, Tirupati, India-517502

[‡]Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, H-6701 Szeged, P.O. Box 521, Hungary

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The response of *Spirulina (Arthrospira) platensis* to high salt stress was investigated by incubating the cells in light of moderate intensity in the presence of 0.8 M NaCl. NaCl caused a decrease in photosystem II (PSII) mediated oxygen evolution activity and increase in photosystem I (PSI) activity and the amount of P700. Similarly maximal efficiency of PSII (Fv/Fm) and variable fluorescence (Fv/Fo) were also declined in salt-stressed cells. Western blot analysis reveal that the inhibition in PSII activity is due to a 40% loss of a thylakoid membrane protein, known as D1, which is located in PSII reaction center. NaCl treatment of cells also resulted in the alterations of other thylakoid membrane proteins: most prominently, a dramatic diminishment of the 47-kDa chlorophyll protein (CP) and 94-kDa protein, and accumulation of a 17-kDa protein band were observed in SDS-PAGE. The changes in 47-kDa and 94-kDa proteins lead to the decreased energy transfer from light harvesting antenna to PSII, which was accompanied by alterations in the chlorophyll fluorescence emission spectra of whole cells and isolated thylakoids. Therefore we conclude that salt stress has various effects on photosynthetic electron transport activities due to the marked alterations in the composition of thylakoid membrane proteins.

Keywords: D1 protein, Energy transfer, Photosynthetic electron transport, Salt stress, Thylakoid membrane proteins

Introduction

Environmental factors such as temperature, UV-light, irradiance, drought and salinity are known to affect photosynthesis in both cyanobacteria and plants. In cyanobacteria, several studies have been conducted on photosynthetic electron transport activities both under salt and high light stress conditions in whole cells as well as thylakoid membranes. In *Synechococcus* cells, NaCl at 0.5 M concentration inactivated both PSII and PSI due to the changes in K/Na ratio (Allakhverdiev *et al*., 2000). In *Spirulina platensis*, salt stress caused decrease in the PSII electron transport by increasing the number of the Q_B-non-reducing reaction centers (Lu and Vonshak, 1999). Recently, Lu and co-workers (Lu and Zhang, 2000; Lu and Vonshak, 2002) have been reported that salt stress itself has no direct effect on PSII activity in *Spirulina platensis* preincubated in the dark, but the same salt stress in combination with PAR (Photosynthetically active radiation) led to a block of electron transport between Q_A and Q_B (primary and secondary quinone electron acceptors of PSII, located in D2 and D1 proteins respectively), and the inhibition of PSII electron transport was proportional to the intensity of light. It has also been revealed that cyclic electron flow around PSI was enhanced during salt stress in cyanobacteria (Gilmour *et al*., 1982; Joset *et al*., 1996).

Several studies demonstrated that thylakoid membrane proteins were affected by salt stress. In higher plants, the 23-kDa protein, which is extrinsically bound to PSII was dissociated under salt stress (Kuwabara and Murata, 1983; Murata *et al*., 1992). D1 protein of thylakoid membranes was showed as a sensitive protein to environmental stress conditions: under various unfavorable conditions like drought, nutrition deficiency, heat, chemical stress, ozone fumigation as well as UV-B and visible light stresses can influence the turnover of D1 protein (see review, Giardi *et al*., 1997). In *Spirulina platensis* it has been proposed that salt stress in combination with moderate light intensities could damage the D1 protein (Vonshak *et al*., 1996). Recently Allakhaverdiev *et al*. (2002) demonstrated salt stress inhibits the repair of photodamaged PSII by concealing the synthesis of D1 protein in *Synechocystis*. In higher plants the susceptibility of PSII to

*To whom correspondence should be addressed.
Tel: 91-877-2249666 Ext 295; Fax: 91-877-2248496
E-mail: sdsmurthy@lycos.com

high light stress (photoinhibition) has been well established, and associated with the degradation of the D1 protein (Aro *et al*., 1993).

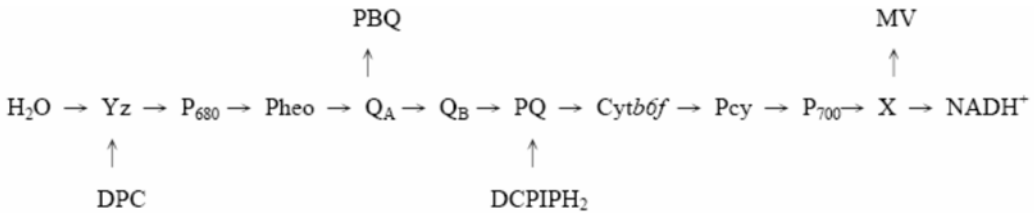
Although the physiological processes involved in salt adaptation of cyanobacteria have been well studied (Schubert and Hagemann, 1990; Jeenjean *et al*., 1993; Zeng and Vonshak, 1998; Lu and Vonshak, 1999; Allakhverdiev *et al*., 2000; Pogoryelov *et al*., 2003), less work has been done with respect to the salt stress conditions on thylakoid membrane proteins, particularly on D1 protein. Thus in the present study, by using a cyanobacterial system, we compared the inhibition of PSII activity under salt stress and the D1 protein content and we report that salt stress at moderate light intensity leads to the damage of D1 protein and alters the fluorescence emission profile of Chl *a*-protein complexes of the thylakoid membranes.

Materials and Methods

Spirulina platensis grew in sodium rich (150-200 mM Na^+) lakes and ponds. The cultures were obtained from Central Food Technological Research Institute (CFTRI), India. *Spirulina platensis (strain Moyse)* cells were grown at 30°C in Zarrouk medium (Zarrouk, 1966) under the continuous illumination (80 µmole photons m^{-2} s^{-1}). The culture was continuously bubbled with filtered air. The mid-log-phase cyanobacterial cells (5 µg Chlorophyll a (Chl a) ml^{-1}) were taken in fresh culture medium and salt treatment was given with 0.8 M NaCl at 80 µmole photons m^{-2} s^{-1}.

Isolation of thylakoid membranes Thylakoid membranes were isolated according to Hagio *et al*. (2000); and Nishiyama *et al*. (1993) with some modifications. *Spirulina* cells were collected from 1 l culture with a cell density at 5 µg Chl a ml^{-1} by vacuum filtration of the cell suspension on a filter paper. The collected cells were washed with buffer A (100 ml of 25 mM N-[2-Hydroxy-ethyl]piperazine-N'-[2-ethane sulphonicacid] (HEPES) -NaOH containing 30 mM $CaCl_2$, pH 7.5) and suspended in 10 ml of buffer B (25 mM HEPES-NaOH, 30 mM $CaCl_2$, and 0.8 M sorbitol containing 1 mM α-toluenesulfonyl fluoride, pH 7.5). The cells were disrupted by sonication at 4°C, at an amplitude of 15 µm with 20 bursts of 15 s each, in MSE sonicator (USA). Thylakoids were separated from the cell debris by centrifugation at 9,000 × *g* for 5 min. The supernatant was again centrifuged at 40,000 × *g* for 90 min at 4°C. The pellet was resuspended in buffer B and utilized.

Oxygen polarography The electron transport activities were measured by using a Hansatech DW2 O_2 electrode at a light intensity of 400 µmole photons m^{-2} s^{-1}. Typically 15 µg Chl *a* ml^{-1} of whole cells suspension or 10 µg Chl *a* ml^{-1} of freshly prepared thylakoid membranes were used in each measurement. For whole-chain electron (WCE) transport activity, the reaction medium (25 mM HEPES-NaOH buffer, pH 7.5, 20 mM NaCl) was supplemented with 0.5 mM MV (methylviologen) and 1 mM sodium azide. PSII activity was measured in the same reaction medium in the presence of 0.5 mM p-BQ (p-benzoquinone). PSI catalyzed electron transport activity was assayed with 0.1 mM DCPIP (2,6-dichlorophenol indophenol), 5 mM ascorbate, 1 mM sodium azide, 10 µM DCMU [3-(3',4'-dichlorophenol)-1,1-dimethylurea] and 0.5 mM MV. The electron transport from PSII to MV was measured by using 0.5 mM DPC (diphenylcarbazid). The Chl a content of the samples was estimated according to MacKinney (1941). Following is the diagrammatic representation of photosynthetic electron transport measurements. MV and p-BQ were used as electron acceptors from PSI and PSII



respectively where as DPC and DCPIPH2 were used as electron donors to PSII and plastoquinone (PQ) pool respectively. DCMU acts as electron inhibitor between Q_A and Q_B (Sudhir and Murthy, 2004).

P 700 content The relative amount of P700 in isolated thylakoids was determined from the difference spectra of oxidized (ferricyanide, 2 mM) *minus* reduced (ascorbate, 5 mM) spectra, according to Hiyama and Ke (1972), using Shimadzu 3000 spectrophotometer.

Fluorescence measurements (i) Room temperature fluorescence emission spectra were measured with PTI (Photon Technology Instruments) 710 spectrofluorimeter. Excitation wavelength was set at 440 nm and slit widths were adjusted to 10 nm (excitation) and 5 nm (emission). 25 mM HEPES-NaOH buffer pH 7.5 containing 20 mM NaCl was used for the measurements.

(ii) Induction kinetics of Chl *a* fluorescence were measured with pulse-modulated fluorimeter (PAM 101, Heinz Walz GmbH, Effeltrich, Germany). Fo and Fm indicate the initial and maximum florescence respectively. The difference between Fm and Fo gives the Fv, variable fluorescence. Fm/Fv and Fv/Fo indicate the efficiency of PSII in primary photochemical reactions. Fo is the minimum fluorescence level in the dark, the F_m level of fluorescence was recorded during 1 s saturating white light pulses obtained from a halogen lamp (KL-1500 Electronic, Schott Glasswerke, Wiesbaden, Germany).

SDS-PAGE analysis and Western blot Thylakoid membrane proteins were separated by SDS-PAGE according to Laemmli (1970) in a 12.5% acrylamide gel containing 2 M urea. Equal Chl a concentration was applied on each well. Comassee stained gels were analyzed by Eagle Eye II gel documentation system, One-

Dscan using Stratagene software. The experiments were done on three different samples and each time the density of the each protein is same and SD is less than 5%.

Western blot was performed according to Towbin *et al.* (1979). The D1 protein was probed with rabbit polyclonal antibody raised against *Synechocystis* PCC 6803 D1 protein. The primary antibody was detected by using goat anti-rabbit IgG secondary antibody conjugated with alkaline phosphatase (Bio-Rad, Richmond, USA). The images of the immunodecorated Nitrocellulose membranes were created and analyzed using the above mentioned system software. After background subtraction the densities were measured on the total area of each visualized protein bands.

Results and Discussion

The present study has demonstrated that salt stress (0.8 M NaCl) inhibited PSII activity whereas stimulated electron transport activity of PSI. The incubation of *Spirulina* cells with 0.8 M NaCl resulted in decreased oxygen evolution activity: the activity of PSII ($H_2O \rightarrow pBQ$) in whole cells decreased gradually to 60% of control during the first 9 hrs of salt treatment (Fig. 1A) and then remained at that level for at least 15 hrs. The maximum efficiency of PSII (Fv/Fm) and variable fluorescence (Fv/Fo) were also measured in whole cells. As shown in Fig. 1A, salt stress caused a 40% loss in F_v/F_m and 54% loss in F_v/F_o. In unreported experiments we have observed that the time course decline of the maximal efficiency of PSII, F_v/F_m, was similar to that of the as whole chain electron transport activity ($H_2O \rightarrow MV$). In order to find out the inhibition site in electron transport, thylakoid membrane proteins were isolated and all the photosynthetic electron transport activities were measured. Fig. 1B shows the activities measured in thylakoid membranes isolated from 9 hrs salt treated cells and control cells. This revealed that whole chain electron transport activity ($H_2O \rightarrow MV$) is inhibited to the same extent as PSII activity. This inhibition could not be restored by diphenylcarbazide (DPC), an artificial electron donor to PSII ($DPC \rightarrow MV$). PSII activity measured with DPC concluded that the site of inhibition is at acceptor side of PSII. In contrast to PSII, the activity of PSI was increased by about 20% ($DCPIPH_2 \rightarrow MV$), the amount of P700 also increased to a similar extent in the thylakoids isolated from salt treated cells relative to the control thylakoids (Fig. 1B).

Since the salt stress caused inhibition in PSII activity, we analyzed the polypeptide profiles of thylakoid membranes to identify the alterations in PSII reaction center proteins as well as other proteins. SDS-PAGE analysis of thylakoid membrane proteins isolated from untreated and salt-stressed cells was showed in Fig. 2. The most prominent salt-stress induced alterations include the decrease in the amount of 34, 47, and 94-kDa proteins, and the appearance of a 17-kDa protein. Previously, the loss in 34-kDa protein under high light stress in *Spirulina platensis* and in several other studies has been accounted for the damage of D1 protein. Thus we have performed Western blot analysis to study the loss in 34-kDa protein and observed a 40% loss as showed in Fig. 1A and 3. The loss in D1 protein has been accounted for the inhibition in PSII activity and also the observed decline in Fv/Fm and Fv/Fo was caused by the degradation of D1 protein. Similar conclusions have been reached in higher and lower plants during photo-inhibitory stress (Ohad *et al*., 1984; Rintamaki *et al*., 1994).

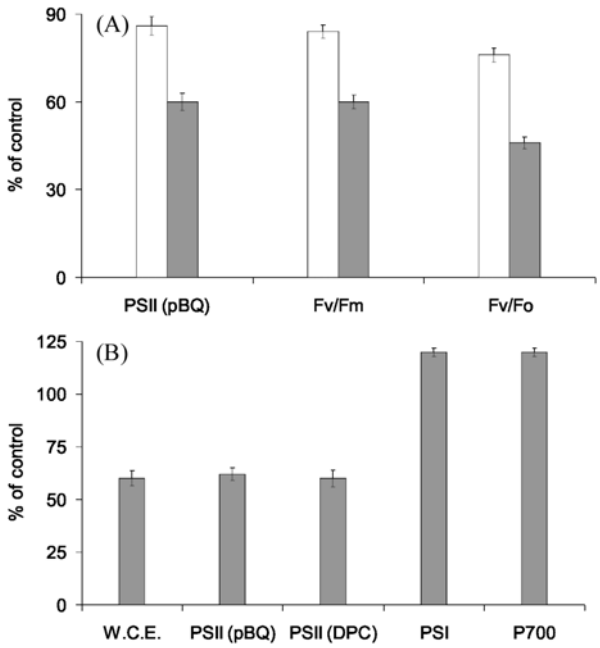


Fig. 1. (A) Effect of salt stress (0.8 M NaCl) on PSII activity, F_v/F_m and F_v/F_o measured in *Spirulina* cells. The values represented in relative units (open and full columns, 3 h and 9 h salt treated cells respectively). In control cells, the PSII activity was 415 ± 13 μmole (O_2 evolved) mg^{-1} (Chl) h^{-1}; F_v/F_m and F_v/F_o were 0.44 ± 0.01 and 0.8 ± 0.019 respectively. Results represented from 3 independent experiments. (B) Effect of salt stress (0.8 M NaCl, 9 hrs) on the electron transport activities and the number of P700 measured in isolated thylakoid membranes. The values represented in relative units. In the control, whole chain electron transport (W.C.E.) activity ($H_2O \rightarrow MV$) was 85 ± 3 μmole (O_2 consumed) mg^{-1} Chl h^{-1}; PSII activity ($H_2O \rightarrow pBQ$ and $DPC \rightarrow MV$) was 290 ± 9 μmole (O_2 evolved) mg^{-1} Chl h^{-1} and and 80 ± 3 μmole (O_2 consumed) mg^{-1} Chl h^{-1}; The PSI activity ($DPCIPH_2$-MV) was 980 ± 24 μmole (O_2 consumed) mg^{-1} Chl h^{-1}; P 700 content was 13.7 ± 0.31 nmole mg^{-1} Chl. Results represented from 3 independent experiments.

Phycobiliproteins and chrorophyll proteins act as light harvesting antenna and transfer the absorbed energy to PSII reaction center, which is essential to initiate the electron transport process. We observed that under salt stress, there is also a loss in the chlorophyll protein (47-kDa) and a core membrane linker-protein (94-kDa) that can attach phycobilisomes to thylakoids (Garnier *et al*., 1994). The changes in 47-kDa chlorophyll protein and 94-kDa proteins were manifest in the alterations in the room temperature fluorescence emission spectra (Fig 4A). In comparison with the control cells, the

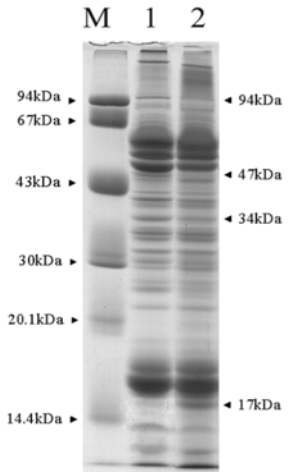


Fig. 2. SDS-PAGE polypeptide profile of thylakoid membranes isolated from control and 9 h salt treated cells of *Spirulina platensis*. Lane M represents marker proteins, Lane 1 and 2 represent thylakoids isolated from control and 9 hrs salt treated cells respectively. Samples were loaded on equal Chl a (12 μg) basis. Other details were given in the materials and methods section.

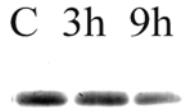


Fig. 3. Effect of salt stress on D1 protein of the PSII reaction center complex determined by Western blot analysis. C represents the D1 content of control cells where as 3 h and 9 h shows the D1 content after 3 h and 9 h of salt stress. The experiments were repeated three times with different samples and the SD is less than 5%.

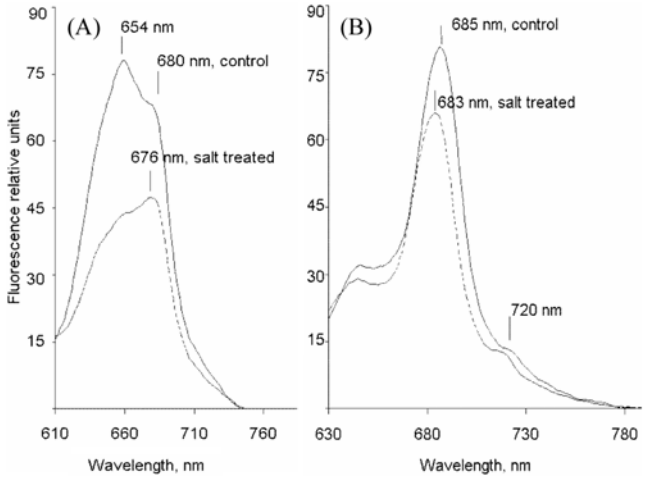


Fig. 4. (A) Room temperature fluorescence emission spectra of intact cells. (—) represents control cells and (-----) represents salt (0.8 M NaCl, 9 hrs) treated cells. The fluorescence spectra were recorded on equal Chl a basis, 4 μg ml^{-1}, excitation wavelength, 440 nm. These experiments were repeated three times and yielded identical spectra, a typical spectrum is presented. (B) Room temperature fluorescence emission spectra of isolated thylakoids from control cells (—), and from salt (0.8 M NaCl, 9 hrs) treated cells (-----). The fluorescence spectra were recorded on equal Chl a basis, 4 μg ml^{-1}, excitation wavelength, 440 nm. These experiments were repeated three times and yielded identical spectra, a typical spectrum is presented.

emission intensity at 654 nm originating from the phycobilisomes was reduced in the salt-stressed cells. The main emission band around 680 nm, which originates from Chl *a* was blue-shifted 4 nm. In the thylakoids from salt treated cells, F685 peak shifted to 683 nm (Fig. 4B). These alterations in fluorescence spectral properties suggest that salt stress resulted in the decreased energy transfer from light harvesting antenna to PSII. Similarly, the alterations in Chl *a* emission properties reflect either the interruption of energy transfer from phycobilisomes to Chl *a* or the loss of CP47 protein as has been suggested by Rajagopal *et al*. (2000) in UV-B exposed *Spirulina* cells and Murthy *et al*. (1995) under mercury ion stress in the *Synechococcus* cells. This type of changes in the Chl *a* emission properties have also been reported in the salt adapted *Spirulina* cells by Verma and Mohanty (2000). In-addition the appearance of 17-kDa protein band in the thylakoids isolated from salt stressed cells, might have been assigned to originate either from the degradation of larger molecular weight proteins or from *de novo* synthesis induced by salt stress. Besides the appearance of 17-kDa-protein band, we also observed the decrease in the intensity of a protein band just below 43-kDa and two protein bands between 20 and 30-kDa. The characterization of the appearance and decreased intensity of these observed unknown proteins needs to be carried-out in future. Similarly, major changes in the protein synthesis and alterations in the protein content after salt stress in *Synechocystis* have been previously reported by Hagemann *et al*. (1990) and Bhagwat and Apte (1989).

Our results show that PSI also affected under salt stress: the observed increase in PSI activity and the number of P700 reaction centers is probably induced by the necessity to maintain ATP pool via cyclic photophoshorylation for Na^+ extrusion from cytoplasm. Similar effects of salt stress on PSI activity and the number of P 700 reaction centers have been observed in *Synechocystis* PCC6803 when exposed to salt stress (Schubert and Hagemann, 1990; Jeanjean *et al*., 1993).

In summary, our results suggest that exposure of *S.platensis* to salinity stress inhibits PSII activity and affects the energy transfer processes by inducing alterations in thylakoid membrane proteins.

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