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Sodium Dependency of the Photosynthetic Electron Transport in the Alkaliphilic Cyanobacterium *Arthrospira platensis*

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Arthrospira (Spirulina) platensis (A. platensis) is a model organism for investigation of adaptation of photosynthetic organisms to extreme environmental conditions: the cell functions in this cyanobacterium are optimized to high pH and high concentration (150–250 mM) of Na^+. However, the mechanism of the possible fine-tuning of the photosynthetic functions to these extreme conditions and/or the regulation of the cellular environment to optimize the photosynthetic functions is poorly understood. In this work we investigated the effect of Na-ions on different photosynthetic activities: linear electron transport reactions (measured by means of polarography and spectrophotometry), the activity of photosystem II (PS II) (thermoluminescence and chlorophyll *a* fluorescence induction), and redox turnover of the cytochrome b_6f complex (flash photolysis); and measured the changes of the intracellular pH (9-aminoacridine fluorescence). It was found that sodium deprivation of cells in the dark at pH 10 inhibited, within 40 min, all measured photosynthetic reactions, and led to an alkalinization of the intracellular pH, which rose from the physiological value of about 8.3–9.6. These were partially and totally restored by readdition of Na-ions at 2.5–25 mM and about 200 mM, respectively. The intracellular pH and the photosynthetic functions were also sensitive to monensin, an exogenous Na^+/H^+ exchanger, which collapses both proton and sodium gradients across the cytoplasmic membrane. These observations explain the strict Na^+-dependency of the photosynthetic electron transport at high extracellular pH, provide experimental evidence on the alkalization of the intracellular environment, and support the hypothesized role of an Na^+/H^+ antiport through the plasma membrane in pH homeostasis (Schlesinger *et al*. (1996). *J. Phycol.* **32,** 608–613). Further, we show that (i) the specific site of inactivation of the photosynthetic electron transport at alkaline pH is to be found at the water splitting enzyme; (ii) in contrast to earlier reports, the inactivation occurs in the dark and, for short periods, without detectable damage in the photosynthetic apparatus; and (iii) in contrast to high pH, Na^+ dependency in the neutral pH range is shown not to originate from PSII, but from the acceptor side of PSI. These data permit us to conclude that the intracellular environment rather than the machinery of the photosynthetic electron transport is adjusted to the extreme conditions of high pH and high Na^+ concentration.

KEY WORDS: Alkaliphile; *Arthrospira (Spirulina) platensis*; cyanobacteria; pH homeostasis; photosynthesis; sodium/proton antiport.

INTRODUCTION

A number of studies have revealed the role of sodium in the metabolism of cyanobacteria. While it has been well established that sodium concentrations exert severe and adverse effects on the photosynthetic apparatus (see, e.g., Lu and Vonshak, 2002; Schubert and Hagemann, 1990; van Thor *et al*., 2000), less attention is paid to the effect of sodium depletion. Most cyanobacterial species require sodium in millimolar or submillimolar

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concentration range to maintain viability especially at alkaline pH (Buck and Smith, 1995; Espie *et al*., 1988; Miller *et al*., 1984). In cyanobacteria incubated in sodium-deficient media, some reports indicated the loss of the photosynthetic oxygen evolution (Brown *et al*., 1990; Espie *et al*., 1988; Miller *et al*., 1984; Zhao and Brand, 1988), degradation of the thylakoid membranes (Avendano *et al*., 1989) and decrease in the content of both chlorophyll and phycobiliproteins (Maeso *et al*., 1987). In *Anabaena* and *Nostoc* the effect of sodium deficiency has been attributed to the pH-dependent inhibition of bicarbonate transport (Kaplan *et al*., 1994; Maeso *et al*., 1987), and could be eliminated by saturation of the cells with CO_2 at near neutral pH (Maeso *et al*., 1987). However, in some other species, sodium appears to exert a more direct effect on the photosynthetic apparatus. In *Synehococcus (Anacystys nidulans)* and in *Synechocystis* PCC 6714, the effect of sodium depletion at high pH could not be accounted for by a limitation in the inorganic carbon substrate. In these cyanobacteria, alterations have been observed in the Chl antenna, and the inhibition of the electron transport upon Na-depletion was attributed to a specific damage at a site near PSII, which could be reversed by the readdition of Na^+ or Ca^{2+} (Zhao and Brand, 1988, 1989).

The effect of sodium deprivation, among the studied cyanobacteria, is most dramatic on *A. platensis*. In this obligate alkaliphilic cyanobacterium a strict Na dependence has been demonstrated for the growth and photosynthetic activity of the cells (Schlesinger *et al*., 1996). It has been shown that not less than 50 mM Na^+ in the medium is required to maintain the growth of *A. platensis* at high pH; Na^+ depletion provoked a light-dependent inhibition of PSII activity, and also led to a loss in the phycocyanin content of cells (Schlesinger *et al*., 1996). It has also been shown that Na^+ provided some protection against the inhibition of oxygen evolution and cell disintegration induced by the addition of uncouplers (Schlesinger *et al*., 1996). On the basis of these observations and on the well-established role of Na^+ and Na^+/H^+ exchange in alkaliphilic heterotrophic bacteria (Krulwich, 1995) it has been proposed that pigment bleaching and the loss of PSII activity are due to the inability of the cells to maintain an appropriate intracellular pH (Schlesinger *et al*., 1996). However, the intracellular pH was not measured and thus no correlation was established between the variations in the photosynthetic activities and the intracellular pH; also, some pH-independent role of Na-ions on the electron transport could not be ruled out. In general, the mechanism of strict Na^+-dependency of the photosynthetic electron transport at high pH, the role of light in the inactivation of photosynthesis in sodium-depleted cells and the sequence of events of restoration are still poorly understood.

In this work, we investigated the photosynthetic electron transport reactions and measured the intracellular pH. Our data provide experimental evidence that in *A. platensis* the main role of Na-ions in the photosynthetic electron transport of *A. platensis* is to maintain, most probably via Na^+/H^+ antiport through the plasma membrane, a pH homeostasis. We also show that the specific site of the inhibition of PSII electron transport is the water-splitting enzyme and, in contrast to an earlier suggestion (Schlesinger *et al*., 1996), inactivation of PSII does not depend on the light. Further we show that it does not involve a damage of the D1 protein. The inactivation of photosynthetic activity, after short depletion periods in the dark, can be fully reversed by readdition of Na-ions to the medium.

MATERIALS AND METHODS

Arthrospira (Spirulina) platensis (strain Moyse) cells were grown at 30°C in Zarrouk's medium (Zarrouk, 1966) under continuous illumination (white light, 60 μmole photons m^{-2} s^{-1}). The culture was continuously bubbled with filtered air. Under these conditions the pH of culture medium was around 9.5. Cells were harvested in mid-log-phase of growth, at a density around 5 μg Chl mL^{-1}. The Chl content of the samples was estimated spectrophotometrically from 95% methanol extractions (de Marsac and Houmard, 1988). All measurements on whole cells were carried out in 0.2 M $KHCO_3$–K_2CO_3 buffer (pH 9.0–10.0), or 20 mM Bis-Tris Propane (pH 7.0–10.0) (Sigma, ultra grade) supplemented with 0.1 M KCl and 0.1 M sorbitol. To obtain the desired sodium concentrations, NaCl was added as required.

For sodium depletion of cells, we used vacuum filtration of the cell suspension on a filter paper followed by resuspension in sodium-free buffer, which ensured that the sodium concentration in the "depleted" cells did not exceed 200–300 μM (estimated from atomic absorption spectroscopy, data not shown). This residual concentration was considerably higher (about 1 mM) when, instead of filtration, we used centrifugation (Ritchie, 1992).

Steady-state rates of oxygen evolution were measured using a Hansatech DW2 O_2 electrode at a light intensity of 400 μmole photons m^{-2} s^{-1}. Typically 5 μg Chl *a* mL^{-1} of whole cells suspension or 10 μg Chl *a* mL^{-1} of freshly prepared thylakoids were used. Chl *a* fluorescence induction kinetics were measured with pulse-modulated fluorometer (Xe-PAM, Heinz Walz GmbH, Effeltrich, Germany). 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).

Thermoluminescence (TL) was measured with computerized laboratory-built apparatus (Demeter *et al.*, 1993). Samples were illuminated at 0°C with white light of 100 μmole photons m^{-2} s^{-1}. The glow curves were recorded from 0 to 65°C, at 20°C min^{-1} heating rate.

Absorbance transients induced by single turnover flashes were measured between 470 and 570 nm by using an equipment described earlier (Büchel and Garab, 1995). The cells were dark-adapted prior to the measurements. The extent of the flash-induced oxidation of cyt*f* was determined from the absorbance change $\Delta A_{\mathrm{cyt}f} = \Delta A_{(554-540\,\mathrm{nm})}$ at 1 ms after the flash.

The intracellular pH was monitored by measuring the fluorescence intensity of 9-aminoacridine (9AA). A simple relation between 9AA fluorescence quenching and ΔpH, with respect to the pH of the medium, and to the intracellular pH, has been proposed for cyanobacterial cells (Blumwald *et al.*, 1984; Teuber *et al.*, 2001); it is based on the assumption that fluorescence quenching is caused mainly by the cytosolic enrichment of the protonated amine. To determine the value of ΔpH we used the model described in Mills (1986). In untreated cells, the dependence of the intracellular pH on the pH of the medium, obtained by this method, was very similar to that determined with the aid of EPR assay (Belkin and Boussiba, 1991).

The thylakoid membranes were isolated according to Hagio *et al.* (2000) and Nishiyama *et al.* (1993) with some modifications. The cells were collected from 1 L culture with a cell density at 5 μg Chl mL^{-1} by vacuum filtration of the cell suspension on a filter paper. The collected cells were washed once with 100-mL 25 mM HEPES–NaOH buffer (pH 7.5) and 30 mM $CaCl_2$ (medium A) and resuspended in 10 mL of medium A containing 0.8 M sorbitol, 1 M glycinebetaine, and 1 mM phenylmethylsulfonyl fluoride (medium B). Cell breakage was performed by sonication (Branson 450 Sonifier, Branson Ultrasonic Corporation, Eagle Rd., Danbury, USA) at 0°C, at an amplitude of 15 μm with 20 bursts of 15 s each. To remove unbroken cells the suspension was centrifuged at 9000 *g* for 5 min at 4°C. The obtained supernatant was centrifuged at 40,000 *g* for 90 min at 4°C. The pelleted thylakoid membranes were resuspended in medium B and stored at −80°C.

Thylakoid membrane proteins were separated by SDS-PAGE according to (Chua, 1980). PAGE was carried out in a 12.5% acrylamide gel containing 2 M urea.

RESULTS

Previous studies have shown that *A. platensis* possesses high photosynthetic activity between pH 9 and 10 in a medium containing about 200 mM Na^+ (Schlesinger *et al.*, 1996). To investigate the pH-dependence of sodium requirement for the photosynthetic electron transport, we measured the oxygen evolution in the presence and absence of Na-ions.

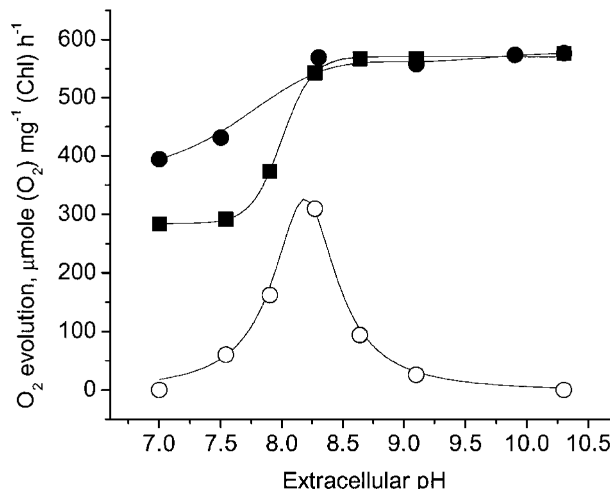

Fig. 1. pH dependency of the net oxygen evolution of *A. platensis* cells in the presence of 200 mM Na^+(●) (control), after sodium depletion (○), and after readdition of 200 mM Na^+ (■) to depleted cells. Sodium depletion and readdition were performed as described in Materials and Methods section. Samples were preincubated for 40 min in the dark in Bis-Tris Propane (20 mM) buffer adjusted to the different pH. Sodium was added in the form of NaCl. Data points represent mean values from five independent series of experiments.

Figure 1 demonstrates that in the presence of 200 mM Na^+ the optimum pH range for the photosynthetic oxygen evolution was between 8.3 and 10.5. The same pH range was found optimal for the growth of the cells, whereas at pH 7 no growth could be observed at any sodium concentration (not shown, cf. Schlesinger *et al.*, 1996). In good agreement with these data, we found that the rate of net oxygen evolution diminished toward the neutral pH in sodium-depleted cells, and a sharp maximum was found at pH 8.3, which was about half of the maximal rate in the presence of 200 mM Na^+. Addition of sodium (200 mM) to depleted cells at pH >8.3 completely restored the oxygen evolution, and partial restoration was observed between pH 7 and 8 (Fig. 1).

These data, is general, are in agreement with the previously reported Na-dependency of the oxygen evolution in *A. platensis* at high pH. However, we must point out that, in contrast to Schlesinger *et al.* (1996), we found that the effect of Na-depletion was not light-dependent, and developed fully in the dark. Schlesinger *et al.* (1996) found that sodium depletion at pH 10 caused complete loss of oxygen evolution in about 60 and 90 min in continuous light of 126 and 40 μmole photons m^{-2} s^{-1}, respectively. In the dark, the incubation in sodium-depleted medium

also led to eventual loss of oxygen evolution, but much longer incubation periods were required. In contrast, in our culture Na-depletion appeared to be complete in the dark in about 40–60 min, and cell lysis was observed only after 6–7 h of incubation. This difference in the response of cells might be due to the difference among the strains used in previous and our study, and/or minor variations in the culture conditions and the preparation of sodium-depleted cells (see Materials and Methods Section). Nevertheless, in unreported experiments we found that light (80 μmole photons m^{-2} s^{-1}) indeed facilitated the inhibition of oxygen evolution, which thus could be achieved in less than 15 min. However, preillumination often interfered with the ability of cells to rapidly retain full activity upon the readdition of sodium.

Although the net rates of oxygen evolution in sodium-depleted cells are equally very small at pH 7 and above pH 9 (Fig. 1) but there is a significant difference between the two inhibitions. In sodium-depleted cells at pH 7 the photosynthetic apparatus was functional, as revealed by thermoluminescence (TL) (Fig. 2) that, similar to the control (pH 10, 200 mM NaCl), exhibited a DCMU-sensitive band, B-band (Vass *et al.*, 1981). The photosynthetic activity of Na-depleted cells at pH 7 has also been confirmed by the flash-induced redox transients of the cytochrome b_6f complex, which displayed essentially the same transients at pH 7 in the absence or presence of sodium, as at pH 10 in the presence of 200 mM NaCl (data not shown). In contrast, as revealed by the same set of experiments, above pH 9 the activities were lost in the absence of sodium.

The above TL data clearly indicate that at least one of the main targets of the Na-depletion at high pH is PSII. To substantiate this conclusion, we performed Chl *a* fluorescence measurement, which carry information on the maximal efficiency of PSII (Campbell *et al.*, 1998). Indeed, the effect of sodium depletion (pH 10) on F_v'/F_m and F_0'/F_m fluorescence parameters was very prominent and fast (Fig. 3(A)). Practically during the first 5 min of sodium depletion of cells in the dark the F_v'/F_m decreased

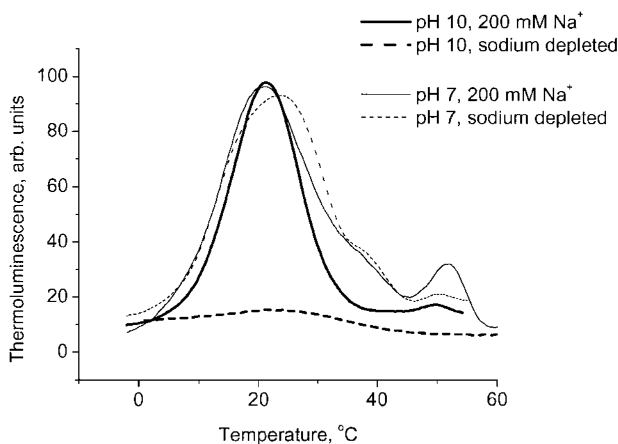

Fig. 2. Thermoluminescence of sodium-depleted (dashed lines) and control (full lines) cells of *A. platensis* at pH 10 (bold lines) and pH 7 (thin lines). The medium for the control cells contained 200 mM NaCl. TL curves were recorded at a heating rate of 20°C min^{-1}, cells were preincubated in the dark for 1 h before the measurements. The cell suspension (500 μL, 25 μg Chl/mL) was excited at 0°C (white light, 100 μmol photons m^{-2} s^{-1}, 20 s).

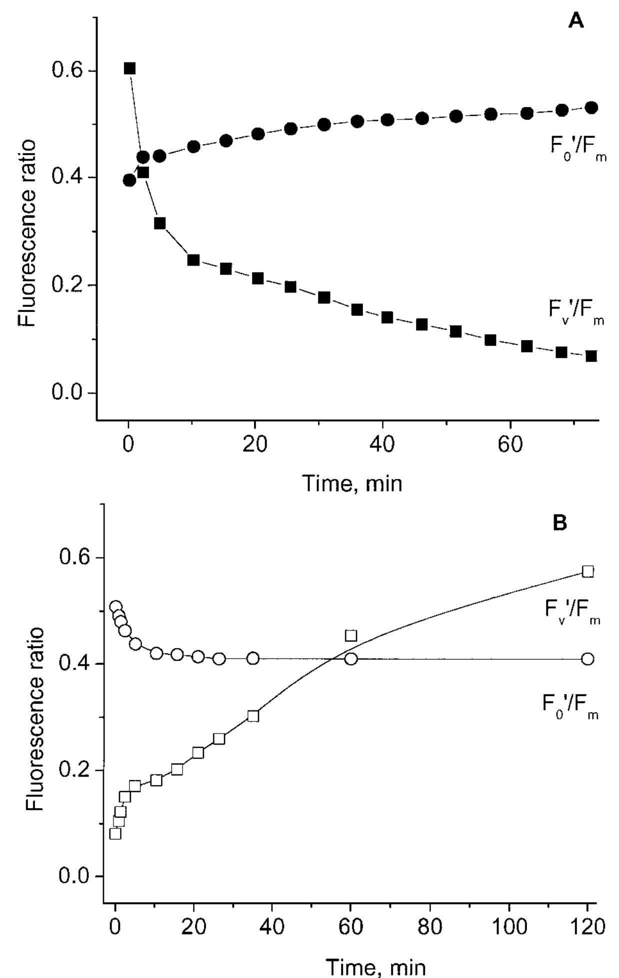

Fig. 3. Changes in the Chl *a* fluorescence parameters F_v'/F_m (■, □) and F_0'/F_m (●, ○) during the depletion of *A. platensis* cells (A, full symbols) and restoration of the cells with 200 mM Na^+ (B, empty symbols). Chl *a* fluorescence was measured with pulse-modulated fluorometer (Xe-PAM). Measuring light was provided by xenon flashes with an interference filter of 440 ± 10 nm, and emission was detected at 695 ± 5 nm. Saturating blue light pulses (1 s, 2000 μmol photons m^{-2} s^{-1}) were obtained from a tungsten source.

to about 50% of the dark-adapted control. As pointed out by Campbell *et al.* (1998), in cyanobacteria this parameter (F'_v/F_m) can be sensitive to the pigment composition. These parameters are influenced by variations in the contribution of the phycobiliproteins to the measured fluorescence. To diminish this uncertainty we applied blue excitation and an observation at 690 ± 5 nm. Thus F'_v/F_m, under these conditions, shows an inhibition of the maximal photochemical efficiency of PSII in sodium-depleted cells at high pH. As shown in Fig. 3(A), sodium depletion also induced significant increase in F_0. This effect was much higher when using PAM 101 (Heinz Walz GmbH, Effeltrich, Germany) that contains a substantial contribution in F_0 from phycobiliproteins. This suggests a detachment of phycobilisomes from the membrane upon Na depletion of cells, which has been confirmed by low-temperature fluorescence spectra (data not shown). The mechanism of this phenomenon, which is also observed at neutral pH, remains unclear.

Addition of 200 mM NaCl restored F'_v/F_m up to about 80% of the dark control in less than an hour and full restoration occurred in 2 h (Fig. 3(B)). Readdition of Na^+ (200 mM) rapidly restored the F'_0 level, which shows that the phycobilisomes can readily be reattached to the membranes.

As shown in Fig. 4(A), in sodium-depleted cells the rate of redox turnover of Cyt f ceased after several dozens of single turnover flashes. As demonstrated by the kinetic traces (inset), the re-reduction was dramatically decelerated after the first few flashes. This is evidently due to a rapid "depletion" of redox equivalents from the intersystem PQ pool, which in turn blocks the turnover of Cyt f upon repetitive flash excitation. These data, and the fact that the "depletion" of redox equivalents was similar upon the addition of 10 μM DCMU (not shown), show that the influx of electrons to the intersystem pool via respiration (Jeanjean *et al.*, 1993) or PSI cycle (van Thor *et al.*, 2000) is not significant.

Readdition of sodium gradually restored the flash-induced oxidation and the dark re-reduction of Cyt f^+; full restoration was observed in about 30 min following the addition of 200 mM Na^+ (Fig. 4(B)).

As summed up in Fig. 5(A), sodium deprivation of the cells at pH 10 resulted, within approximately 15 min, in 80% inhibition of the net photosynthetic oxygen evolution. In the same time, as it was revealed by the time course of 9AA fluorescence, the intracellular pH in Na-depleted cells increased from 8.3 to 9.6.

Compared to these relatively rapid changes, the inhibition of Cyt f turnover was retarded, evidently because of the residual reducing equivalents in the intersystem PQ pool. Upon the addition of sodium, the net photosynthetic oxygen evolution and Cyt f turnover rapidly recovered. The intracellular pH also decreased to a physiological value (8.3), but this lagged somewhat behind the recovery of the photosynthetic activities (Fig. 5(B)).

It seems likely that the Na-dependent variation of the intracellular pH similarly to heterotrophic alkaliphilic bacteria can be explained by the operation of an Na^+/H^+ antiporter. To dissipate both Na^+ and H^+ gradients we applied monensin, an exogenous Na^+/H^+ exchanger. Monensin inhibits the operation of the antiporter because it exchanges the intracellular protons at the expense of extracellular sodium. Indeed, as shown in Fig. 5(C), upon the addition of monensin the intracellular pH raised significantly because of the exchange of extracellular Na^+ with intracellular H^+. Within 20 min it reached a value of about 9, and in 1–2 h the intracellular pH gradually approached the extracellular value (pH 10). This was accompanied by the gradual inhibition of the photosynthetic activities: after 20-min incubation with monensin the oxygen evolution was inhibited up to 90%, while the inhibition of the Cyt f activity lagged again somewhat behind.

As already shown by Schlesinger *et al.* (1996) for optimal growth at pH 10, 200 mM Na^+ is required. However, much lower concentrations are needed for short-term photosynthetic electron transport and for maintaining the intracellular pH within the physiological range. In good agreement with Schlesinger *et al.* (1996) we found that, after sodium depletion of the cells at pH 10 for 35 min, 3 mM Na^+ was necessary to restore the half-optimal rate of the photosynthetic oxygen evolution (Fig. 6). It can be seen that essentially the same concentration of sodium was required to initiate the restoration of the intracellular pH. Much higher concentrations were required (more than 25 mM of Na^+) to complete the restoration of the photosynthetic oxygen evolution and of the intracellular pH. It is important to note that the restoration of these two reactions followed practically the same dependence on the concentrations of Na-ions.

As shown in Fig. 7, in sodium-depleted cells artificial electron donors to PSII, DPC, and Cyt b_6f, DQH_2, fully restored the activities of the linear electron transport and the turnover of the Cyt f, respectively. These data show that the photosynthetic electron transport chain from PSII-Tyr Z to PSI acceptor side remains intact even in sodium-depleted cells at high intracellular pH, and thus the high-pH-sensitive site, similarly to the isolated higher plant membranes (Huber, 1979), is located in the oxygen evolving complex (cf. Bernát *et al.*, 2002).

As shown in Fig. 8, the oxygen evolution of isolated thylakoids of *A. platensis*, similarly to isolated higher plant membranes (Huber, 1979), is also inhibited at high pH, and the maximal rates are found around pH 7.5. Figure 8

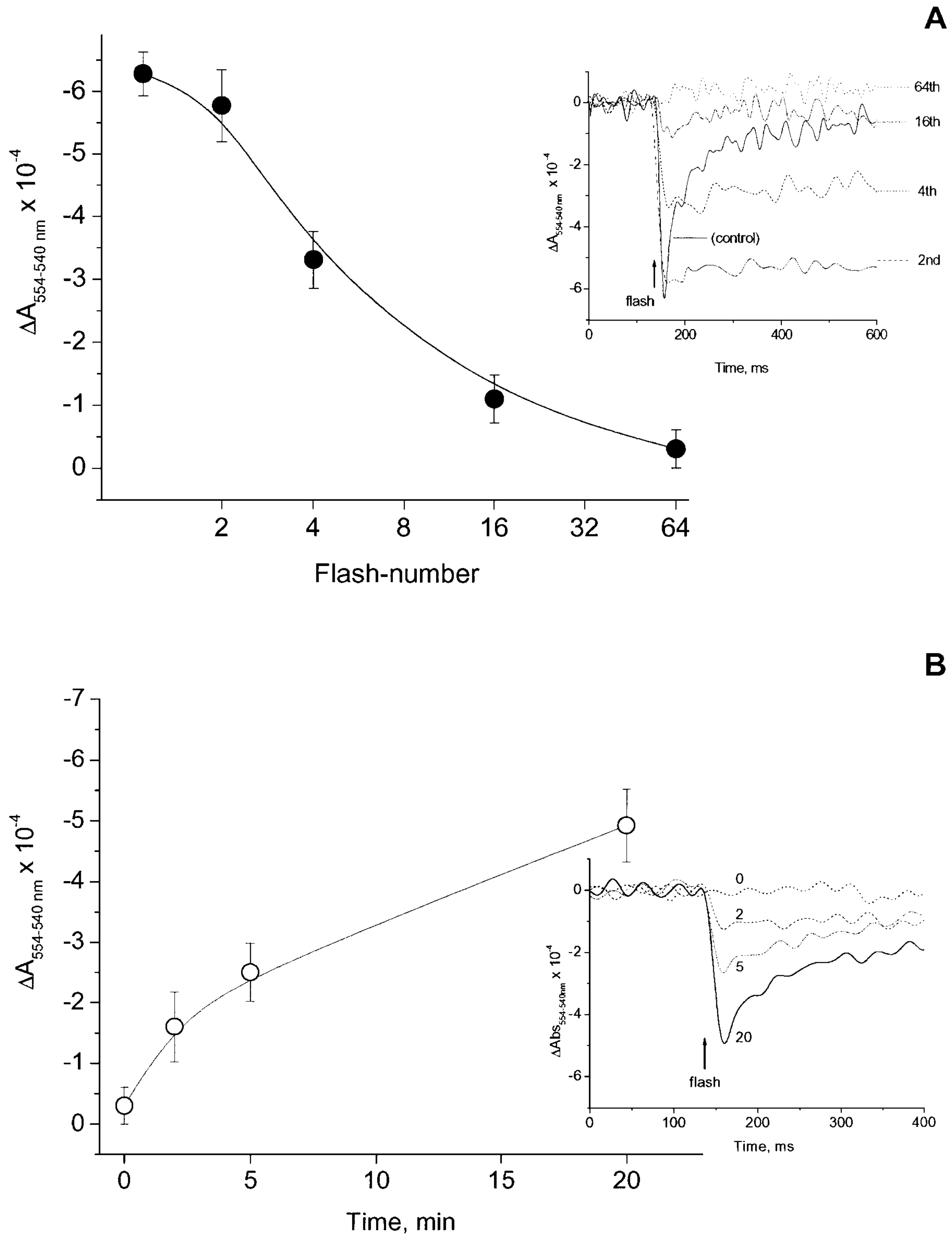

Fig. 4. The initial amplitude of the flash-induced absorbance changes because of the oxidation of Cyt$f$ as a function of the number of single turnover flashes after Na depletion of dark-adapted (20 min) cells (A), and as a function of time after the restoration of cells with 200 mM Na^+ (B). Data points represent mean values ± SEM from three independent sets of experiments. Typical flash-induced absorbance transients of Cyt$f$ are shown in inset. The Chl concentration of the cells was 15 μg Chl mL^{-1}; flash frequency was 1 s^{-1}.

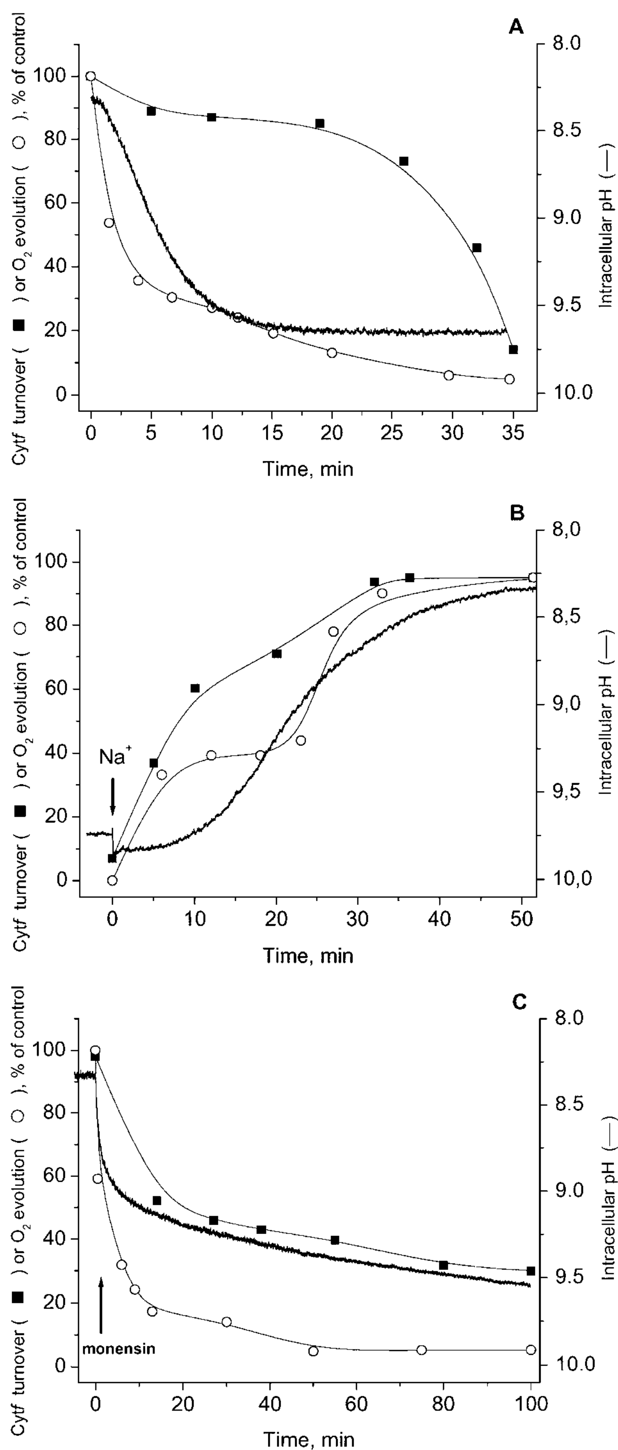

Fig. 5. Time course of the intracellular pH (—), Cyt*f* turnover (■), and net oxygen evolution (○) in *A. platensis* cells following sodium depletion (A) and readdition of 200 mM NaCl (B), and the addition of 20 μM monensin in presence of 200 mM NaCl (C). Typical traces of the 9AA fluorescence due to variations of the intracellular pH; data points on Cyt*f* turnover and oxygen evolution, mean values from three independent sets of experiment.

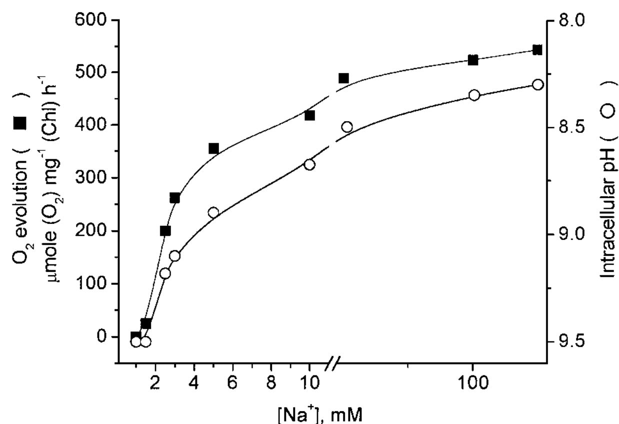

Fig. 6. Dependences of the intracellular pH (○) and of the net photosynthetic electron transport (■) on the concentration of Na^+ applied in the restoration of sodium depletion of *A. platensis* cells. Restoration was performed by the addition of NaCl at different concentrations to cells depleted of sodium for 40 min in the dark.

also shows that in isolated thylakoids of *A. platensis*, the linear electron transport does not depend on the presence of Na-ions (in the millimolar concentration range).

SDS-PAGE profile of thylakoid membrane proteins from sodium-depleted (2 h) cells did not reveal significant alterations in thylakoid membrane protein composition when compared to control cells (Fig. 9). The amount of D1 protein of the PSII reaction center, the most sensitive component of PSII to various stresses in the light (Aro *et al.*, 1993), did not decrease noticeably (not shown). This is in agreement with electron transport data obtained with DPC and MV.

DISCUSSION

A. platensis represents a relatively wide group of the filamentous cyanobacteria (gen. *Oscillatoriaceae*); it is an inhabitant of soda lakes, rich in sodium bicarbonate. It is very intriguing question how this photosynthetic organism is capable of combating two extreme environmental factors, high pH and high concentration of Na^+. In fact, in this particular species the adaptation of cells does not only permit growth at elevated pH, as in the case of many other alkalitolerant cyanobacteria, but the cell functions are optimized to these extreme conditions (pH 9.5–11.5 and 150–250 mM NaCl). In particular, as shown by Schlesinger *et al.* (1996), and in this paper, in the alkaline pH range the photosynthetic electron transport depends strictly on sodium.

As concerns the mechanism of this strict sodium-dependency two stratagems might be "applied": (i) some

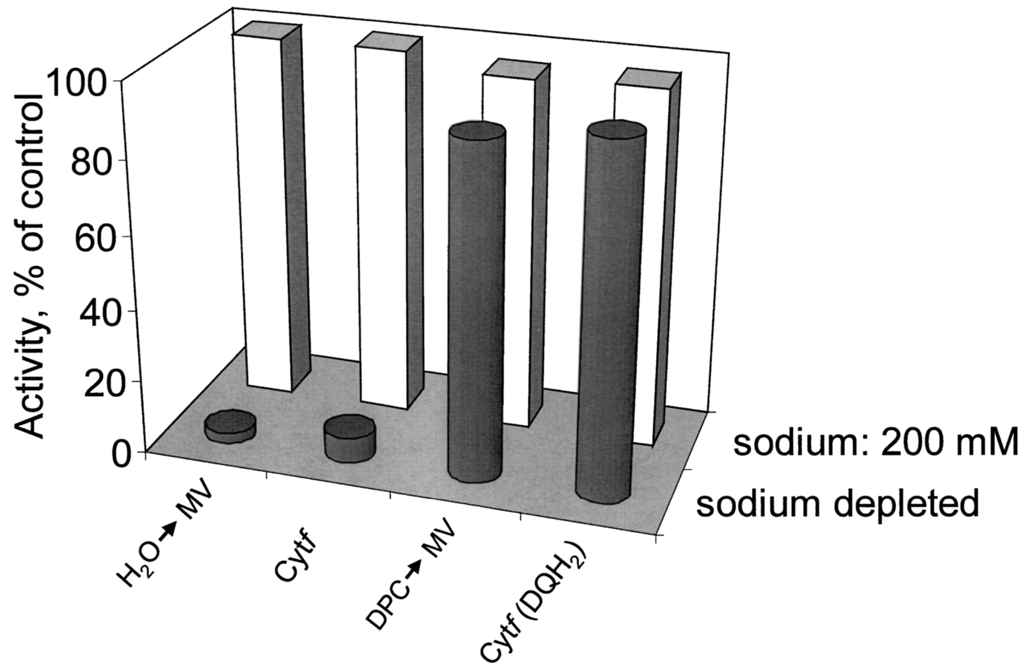

Fig. 7. Electron transport activities in control (200 mM Na^+) and Na^+-depleted cells of *A. platensis*. Linear electron transport rates, $H_2O \rightarrow$ MV and DPC $\rightarrow$ MV were measured with an oxygen electrode (both DPC and MV were added at a final concentration of 1 mM). The turnover of Cyt*f* was determined from the oxidation of Cyt*f* upon excitation with single turnover flashes, measured in both the absence and presence of 10 μM DQH_2.

components of the photosynthetic apparatus undergo alterations because of the unusual conditions (high pH, high salt), i.e. the photosynthetic functions are modified; or

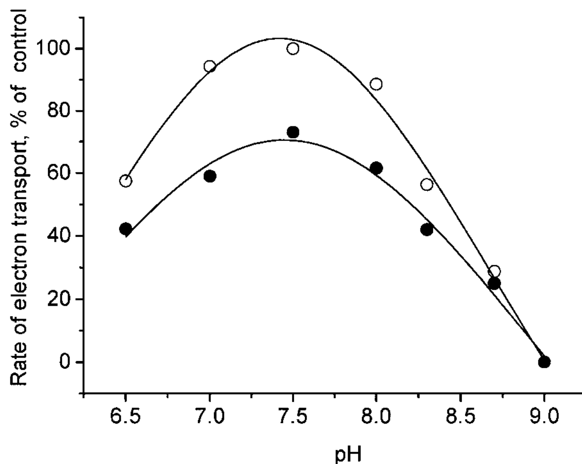

Fig. 8. Dependences of the photosynthetic electron transport rates of isolated thylakoid membranes of *A. platensis* in the absence (○) and presence (●) of 30 mM NaCl. Thylakoids (10 μg Chl/mL) were freshly isolated and suspended in 20 mM Bis-Tris Propane buffer immediately prior to the measurements in the presence of 1 mM MV as an electronacceptor.

(ii) both the intracellular pH and the Na^+ concentration are regulated, i.e. lowered, in order to accommodate the photosynthetic functions.

The fact that deviations from the optimal conditions readily lead to a block in the photosynthetic electron transport would argue for the first scheme. For instance, it is well known that *A. platensis* cannot grow at neutral pH at any sodium concentration (Belkin and Boussiba, 1991). We have also shown that either lowering the pH and/or the sodium concentration can result in severe inhibitions in the measured photosynthetic activities (see Figs. 1 and 6). However, a closer inspection of the data reveals that the mechanisms of inhibition at neutral pH and at high pH are strikingly different.

At high extracellular pH, in the absence of sodium, or in the presence of monensin, inhibitor of the Na^+/H^+ antiport (Fig. 5(C)), the inhibition is clearly correlated with the elevation of intracellular pH, and the site of inhibition is located at the oxygen evolving complex (OEC) (cf. Fig. 7). Earlier, it has been shown that the cells do not grow at pH 7.0 (Schlesinger *et al.*, 1996), which—together with the Na-dependent inhibition of the electron transport at pH 7 (Fig. 1)—implied that similar inhibition might occur at neutral pH. However, our TL data (Fig. 2) revealed that at pH 7 PSII remains fully

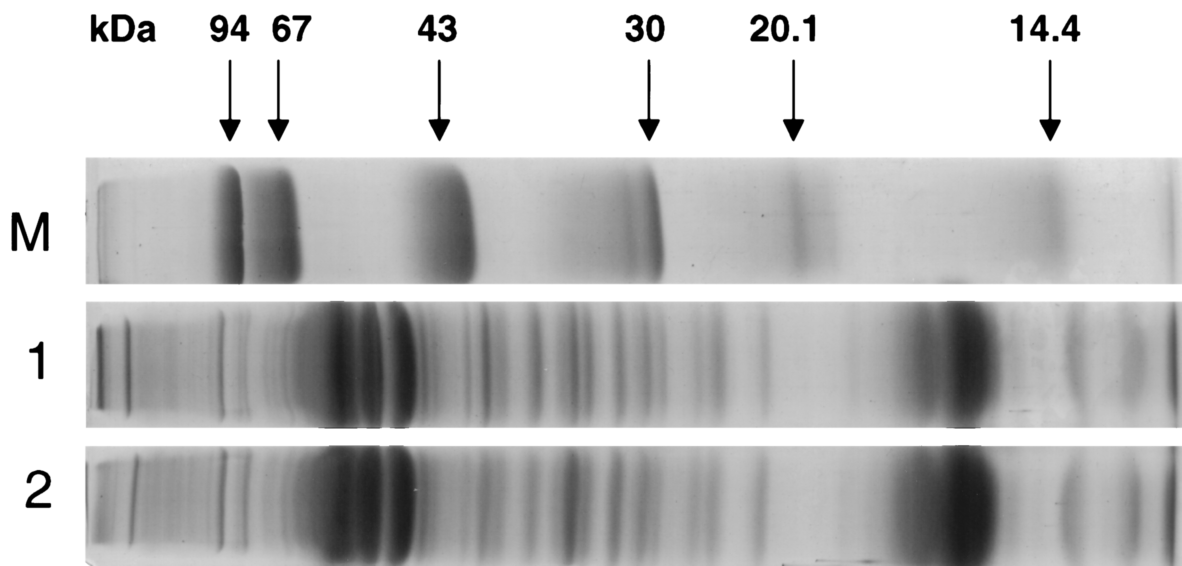

Fig. 9. SDS-PAGE profile of thylakoid mebrane proteins isolated from *A. platensis* cells; lanes 1 and 2, thylakoids from control cells (200 mM Na^+) and sodium-depleted cells (2 h) respectively; M, molecular-weight marker proteins. Samples were loaded on equal Chl (12 μg) basis.

functional. Further, we show that the site of inhibition induced by sodium/depletion must be found at the acceptor side of PSI: the net photosynthetic oxygen evolution from H_2O to CO_2 was rapidly inhibited when the cells were depleted at pH 7, but the linear electron transport rates of $H_2O \rightarrow p$-benzoquinone (PSII) as well as diaminoduren $\rightarrow$ MV (PSI, in the presence of artifical electron acceptor) were not inhibited (data not shown). A sodium-dependent inhibition due to limitation at the acceptor side of PSI can be envisioned if one assumes that *A. platensis* cells rely on the import of HCO_3^- via a Na^+-dependent mechanism. This type of bicarbonate transport has been well established in different cyanobacteria (Kaplan *et al.*, 1984; Ritchie *et al.*, 1996).

Sodium depletion may also affect the PSI electron transport via ferredoxin: $NADP^+$ reductase, which is known to be associated with the phycobilisomes (Schluchter and Bryant, 1992; van Thor *et al.*, 2000). As indicated by Chl *a* fluorescence measurements (Fig. 3), and low temperature fluorescence emission spectra (not shown), phycobilisomes are rapidly detached from the membrane. This effect could, however, be observed in the entire pH range between 7 and 10. The mechanism and physiological significance of this change are not understood—this warrants further investigations. However, as demonstrated in Fig. 7, in the presence of suitable electron donors, the electron transport chain between Tyr-Z (PSII) and the acceptor side of PSI remains fully functional.

At high pH, our data clearly show, that the main site of the inhibition is located at the acceptor side of PSII. Earlier it has been concluded that the "labile photosynthetic component was associated with PSII," and it has been suggested that in the absence of sodium the cells lose their ability to maintain an appropriate internal pH (Schlesinger *et al.*, 1996). However, the intracellular pH was not measured, and no attempt was made to identify the site of inhibition in PSII. Our data show that the specific and sole site of inhibition is at the OEC, and that the inhibition arises indirectly, from the elevation of the intracellular pH upon the depletion of the cells from sodium. It can be ascribed to the effect of high intracellular pH on the S-state transitions, as in chloroplast thylakoids (Bernát *et al.*, 2002). Hence, we not only do not find evidence for any modification of the photosynthetic apparatus by high pH, but in fact we show that a key membrane constituent, the OEC, does not tolerate high pH. Similarly, we can rule out the modification of electron transport by high concentrations (150–250 mM) of sodium. Although we have not measured the intracellular Na^+-concentration, the effect 30 mM sodium on the electron transport rates of thylakoid membranes of *A. platensis* suggests that the intracellular concentration, similarly to the proton concentration, is regulated at the cellular level.

We must stress that the regulation of the intracellular pH (and most likely also the Na^+ concentration) can be achieved in the dark (see Results section). As a consequence, the photosynthetic electron transport is void

of light-induced degradations, which are usually facilitated in combination with stress factors (Giardi *et al*., 1997). These data are at variance with those presented by Schlesinger *et al*. (1996), who found that "the Na^+-deficiency effect was not only pH-dependent but also light-dependent," and observed drastic effects on cell intergity. Under our experimental conditions, no detectable damage was found and the changes, after short periods of sodium depletion in the dark, were fully reversible.

Our observations also show that the photosynthetic electron transport is not directly involved in the homeostasis of cells. The dependence of alkaliphilic heterotrophic bacteria on sodium is well documented (Booth, 1985; Krulwich *et al*., 1999; Padan and Schuldiner, 1994). The central participants in the active cycle that enable prokaryotes to achieve pH homeostasis under alkaline pH is a group of structurally diverse cation/proton antiporters and primary ion pumps that function in different ranges of cation concentrations. While the alkalitolerant bacteria involve the primary sodium pump to drive the electrophoretic H^+ uptake (Skulachev, 1999), the extremely alkaliphilic bacteria rely upon Na^+/H^+ electrogenic antiporter (Krulwich *et al*., 1999, 2001; Padan and Schuldiner, 1994).

Recent studies on *Synechocystis* PCC6803, a cyanobacterium strain, revealed the presence of five putative genes in the genome of this cyanobacterium homologous for Na^+/H^+ antiporters and it has been demonstrates that at least three of these genes (namely *nhaS1*, *nhaS2*, *nhaS3*) encode proteins involved in sodium circulation and low- and high-affinity Na^+/H^+ antiporters in *Synechocystis* PCC6803 (Inaba *et al*., 2001; Mikkat *et al*., 2000). However, the properties of these antiporters have not been studied in details. Nevertheless, the involvement of these antiporters in high-pH tolerance seems to be likely in *Synechocystis* cells.

For *A. platensis* the involvement of Na^+/H^+ antiporter has been proposed by Schlesinger *et al*. (1996); the antiporter might be involved in the regulation of the intracellular pH, similar to the ones found for other alkaliphilic eubacteria (Krulwich *et al*., 1999, 2001). However, the supplementary role of primary Na^+ extrusion systems (also found in some alkaliphilic bacteria; Krulwich *et al*., 2001) could not be ruled out. Our data, by establishing clear correlation between the depletion of Na-ions and the intracellular pH (Figs. 1, 5, and 6), as well as the inhibitory effect of monensin on the intracellular pH (Fig. 5(C)), provide experimental support for the proposed involvement of the Na^+/H^+ antiport in the pH homeostasis of *A. platensis*.

Thus, we conclude that at extremely alkaline pH, the main role of Na-ions in the photosynthetic electron transport of *A. platensis* is to prevent the inactivation of the water-splitting enzyme by maintaining, most probably via Na^+/H^+ antiport through the plasma membrane, a pH homeostasis.

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