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Na^+/H^+ EXCHANGE IN THE CYANOBACTERIUM SYNECHOCOCCUS 6311

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SUMMARY: The cyanobacterium Synechococcus 6311 adapts to grow in 0.6 M NaCl by developing an efficient system for sodium extrusion. In the present investigation cells loaded with NaCl were subjected to a large dilution. Changes in fluorescence quenching of acridine orange as a function of transmembrane Na^+ gradients provide evidence that Na^+/H^+ exchange activity greatly enhanced in salt-adapted cells.

Mechanisms of ion transport are being investigated in the fresh water cyanobacterium Synechococcus 6311, an organism which can adapt to grow in 0.6 M NaCl. The process of salt adaptation includes intracellular accumulation of organic and inorganic osmoregulatory substances and a Na^+ extrusion mechanism [1,2]. When Synechococcus 6311 grown in low salt is suspended in 0.6 M NaCl, there is a marked increase in intracellular sodium content: reading 550 mM after 30 min. exposure to 0.6 M NaCl. This intracellular sodium concentration decreases during growth, stabilizing at 40 mM after 40 hrs. of growth in 0.6 mM NaCl, indicating the presence of an effective sodium extrusion mechanism during adaptation to NaCl [2]. In another cyanobacterium, Nostoc muscorum [3,4], Na^+ seems to have a specific effect in the initiation of events leading to cellular adaptation to high salt. Furthermore, it has been suggested [5] that the active Na^+ extrusion in the cyanobacterium Anacystis nidulans is driven by a Na^+/H^+ antiporter located in the plasmalemma. Hence, it is important to investigate Na^+ transport processes in these cells before and after adaptation.

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Abbreviations: TCS (Tetrachloro salicylanilide), ECCP (Fluorocarbonyl Cyanide, m-chloro-phenylhydrazone), TPT (Triphenyltin), EDTA, (ethylene diamine tetra-acetic acid).

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We report now that transmembrane Na^+ gradients in Synechococcus 6311 induce movement of H^+ suggesting the existence, in that organism, of a Na^+/H^+ exchange system.

METHODS

Synechococcus 6311 cells were grown as described previously [1] in either low salt (11 mM Na^+) or high salt (0.6 M NaCl) growth media. After 96 hours, cells were harvested by centrifugation (10,000xg, 10 min) at 25° C and resuspended in growth medium (pH 7.8). Permeability to NaCl was measured by following osmotic volume changes after NaCl-induced stress using an ESR method for internal cell volume described in [2]. In this method an ESR signal, representing the concentration of rapidly permeable spin probe TEMPONE, is selectively measured inside the cells by quenching the external signal with impermeable paramagnetic ions. Kinetic resolution was achieved using a rapid mixing apparatus [2].

Development of pH gradients in response to outwardly directed Na^+ gradients was followed by the fluorescence quenching of acridine orange [6]. To generate outwardly directed Na^+ gradients, 6 µl of NaCl loaded Synechococcus 6311 cells were diluted in 333-fold solutions containing 1 µM acridine orange (Eastman Kodak, Co.), 0.6 M mannitol and 25 mM N-methylglucamine-gluconate (prepared by titrating solutions of D-gluconic acid lactone with N-methylglucamine) pH 7.8 and concentrations of Na^+ of K^+ salts as indicated. For salt loading cells suspended in their growth medium were sedimented in an Eppendorf centrifuge, resuspended with one volume of growth medium, supplemented with NaCl to the final concentration indicated in the figures, and kept at room temperature in the dark. Final protein concentration was 8.5-9.5 mg/ml (approx. 2 mg chlorophyll/ml). Changes in the fluorescence were monitored with a Perkin-Elmer MPF 44A spectrofluorimeter (excitation, 493 nm; emission, 530 nm), at room temperature. The excitation slit was set at 1 nm to minimize induction of photosynthetic activity.

RESULTS

Comparison of control and salt adapted cells

Control grown (11 mM Na^+) and 0.6 M NaCl grown cells shrink when transferred into 1.0 M NaCl, reaching a minimum volume within 200 ms (Fig. 1). Thereafter, control and 0.6 M NaCl grown cells swell reaching their original volume within 15 and 60 seconds respectively. Elemental analysis of the re-swolen cells (results not shown) confirms that the recovery of volume is due to entry of NaCl. This permeability of cells to NaCl after salt-induced stress suggests that, upon storage in the dark for a few minutes, NaCl equilibration will be significant with either 0.6 or 1.0 M NaCl solutions.

Dilution of cells incubated with either 0.6 M or 1 M NaCl in Na^+-free medium resulted in quenching of acridine orange fluorescence (Fig. 2). Different incubation times of up to 2 hours were tested, but no significant difference

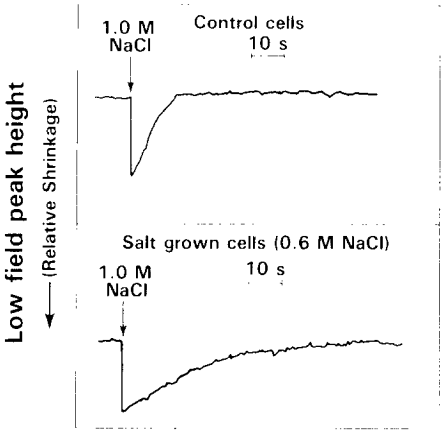

Fig. 1. Kinetics of the osmotic response to NaCl by Synechococcus 6311. Arrows indicate time of mixing with NaCl.

was observed. A fraction of the quenched fluorescence was restored upon addition of 54 mM Na_2SO_4. Recovery of fluorescence, to about the same extent, was obtained by collapsing the transmembrane pH gradient with NH_4Cl or with 0.04 % Triton X-100. A second addition of either Na_2SO_4, NH_4Cl, or detergent failed to induce further increase of the fluorescence signal. These results indicate that the reversible fluorescence quenching was due to the formation of a Na^+ dependent pH gradient. Because the level of fluorescence achieved after addition of Na_2SO_4 was independent of the specific NaCl concentration used for salt-loading of the cells (control and salt-grown), but had a linear rela-

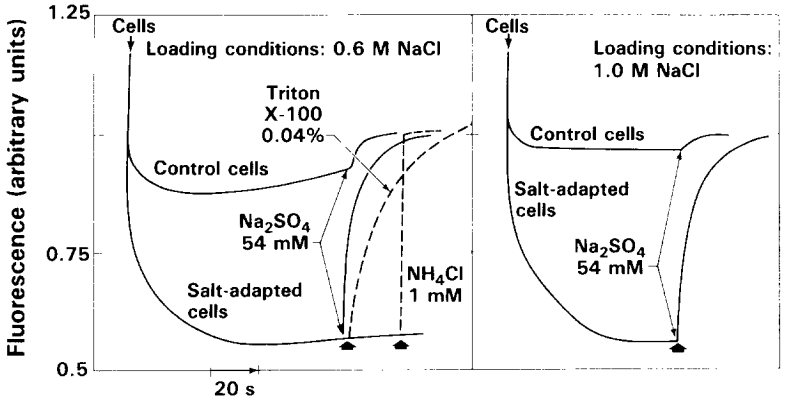

Fig. 2. Quenching of acridine orange fluorescence by dilution of NaCl-loaded Synechococcus 6311 cells into a Na^+-free medium. To start the experiment NaCl loaded cells were diluted into test medium; where indicated Na_2SO_4, NH_4Cl or Triton X-100 0.04% were added.

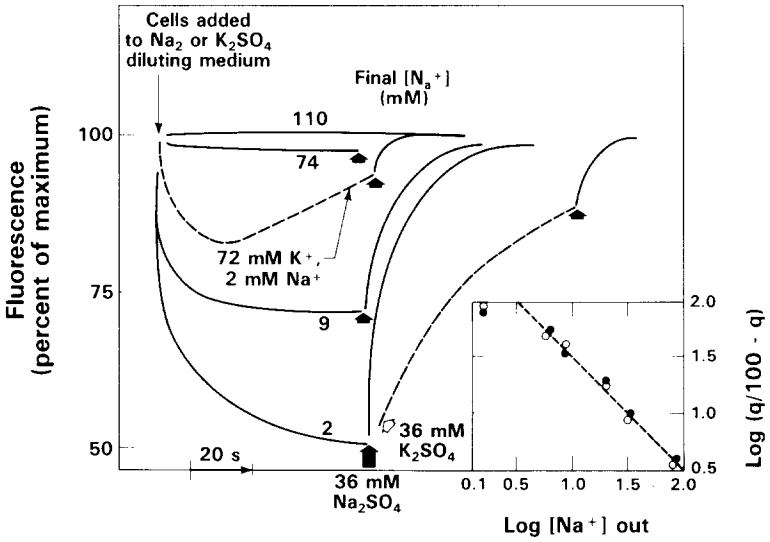

Fig. 3. Quenching of acridine orange fluorescence by dilution of NaCl loaded Synechococcus 6311 cells into a Na^+ or K^+ containing medium. Conditions as in Fig. 2 except salt-adapted cells were used and Na^+ or K^+ was initially present or added later to the reaction mixture. Inset: double log plot of relation between fluorescence and Na^+ concentration in the test medium.

tionship to the amount of cells included in the uptake medium, it was concluded that the irreversible component of the fluorescence quenching resulted from optical opacity and absorbance of the cells. Hence, the fluorescence level achieved (after addition of Na_2SO_4) was scaled to 100% fluorescence (Fig. 3, Table 1). For cells loaded with 0.6 M NaCl the extent of fluorescence quenching attained was 5-fold larger in salt-grown, than in control cells. These differences between control and salt grown cells were even larger when the NaCl concentration in the loading procedure was 1 M.

Similar results (not shown) were obtained using 1 μM 9-amino acridine as the pH probe. However, in this case, quenching due to cell absorbance was three-fold larger than with acridine orange while the Na^+ gradient-sensitive quenching decreased 10-fold. Thus, acridine orange appeared to be a more sensitive probe and was used in subsequent experiments.

Na^+ specificity. Only salt-adapted cells were employed in the subsequent studies. The Na^+-dependence of the extent of fluorescence quenching on the concentration in the diluting medium is shown in Figure 3. The level of

TABLE I

Effect of Ionophores and Medium Acidification on Fluorescence Recovery

Addition	Extent of Fluorescence Recovery Percent
TCS (5uM)	38
FCCP (6uM)	<10
EDTA (0.5uM)	n.d.
TCS + EDTA	72
FCCP + EDTA	42
TPT (1 uM)	91
pH to 7.3	n.d.
pH to 6.6	19
pH to 5.3	90
50 mM Na_2SO_4	100

Cells were added to Na-free medium at pH 7.8 as in Figure 1. After maximum fluorescence quenching, ionophores or acid (H_2SO_4) were added. This was followed 1 min later by 50 mM Na_2SO_4 to induce complete recovery of fluorescence (100 per cent). The values given are the fraction of fluorescence regained 1 min after the indicated additions.

n.d. = non-detectable

fluorescence attained was independent of whether Na_2SO_4 was initially present in the diluting medium or added afterwards. Replacement of Na^+ by K^+ in the diluting medium also resulted in recovery of most of the fluorescence, but the rate of recovery was 6-fold slower with K^+. Moreover, significant signal quenching could still be achieved with high K^+ (72 mM) present in the diluting medium. The rate of recovery of quenched fluorescence was at least 6-fold greater upon addition of Na^+ as compared to K^+. Recovery of quenched fluorescence (i.e., loss of pH gradient) with external K^+ is expected, as introduction of a permeable cation in the medium will facilitate Na^+ release by counter flow, resulting in turn, in loss of the pH gradient.

In a different experimental option to test for specificity, salt-grown cells were washed and suspended in a growth medium in which 0.6 M KCl replaced 0.6 NaCl. After 1 hour of incubation these cells were subjected to the same dilution procedure as described (Methods, Figures 1-2). No significant K^+ gradient dependent quenching of fluorescence was observed.

<u>Na^+/H^+ stoichiometry</u>. A double logarithmic plot of the ratio between quenched (Q) and remaining (100 - Q) fluorescence vs the external Na^+ concentration showed a linear relationship with a slope of 1 (Fig. 3, inset). A 1:1

stoichiometry of Na^+/H^+ exchange is indicated by the data (Fig.3). The relationship obtained for the ratio of quenched to remaining fluorescence and the external Na^+ concentration can be explained as follows: equilibration of Na^+ with H^+ is achieved when $[H^+]_{in}/[H^+]_{out} = [Na^+]_{in}/[Na^+]_{out}$ (I). For a weak base monoamine such as acridine orange (pK 10), whose unprotoned form only is permeable, it holds that $[A]_{in}/[A]_{out} = [H^+]_{in}/[H^+]_{out}$ [11,12], where $[A]_{in}$ and $[A]_{out}$ are the free (unbound) dye concentrations in the intracellular and extracellular aqueous compartments respectively. The total amine inside the cell equals $[A]_{in}x(n+1)xV_{in}$, where n is the number of bound dye molecules for each free molecule and V_{in} is the intracellular volume. For acridine orange this quantity has been shown to correlate with the percent of fluorescence quenching, Q, observed in response to a transmembrane H^+ gradient [12,13]. Thus, 100 - Q represents the amount of unaccumulated dye, $[A]_{out}xV_{out}$, where V_{out} is the extracellular volume. Therefore $Q/(100 - Q) = (1+n)xV_{in}x[H^+]_{in}/V_{out}x[H^+]_{out}$. Given relationship (I), a double logarithmic plot of $Q/(100 - Q)$ versus $[Na^+]_{out}$ will yield the observed straight line with a slope of 1, provided that $[Na^+]_{in}$ remains nearly constant, i.e., only a small fraction of preloaded Na^+ is dissipated on H^+ accumulation. Deviation for low $[Na^+]_{out}$ might be due, among other reasons, to depletion of $[Na^+]_{in}$ since to reach equilibrium, more H^+ needs to be accumulated and more Na^+ has to be extruded. The resilience of the quenched signal to acidification of the medium (Table 1) suggests that significant Na^+ concentrations were still present in the cellular compartment relevant for Na^+/H^+ exchange.

Effect of ionophores. To gain further insight into the properties of the Na^+/H^+ exchange activity, the effect of ionophores and changes in external pH on the recovery of quenched fluorescence was studied (Table 1). Addition of 6 μM FCCP or 5 μM TCS, H^+ ionophores, induced gradual and limited signal recovery. No further increases in recovery rate were obtaind using higher ionophore concentrations. The H^+-ionophore, however, became more effective when EDTA was added to the medium. The EDTA effect was immediate, suggesting that its action may be localized at the extracellular surface. TPT, a Cl^-/OH exchange iono-

phore, induced almost complete fluorescence recovery. Changing the medium pH to 7.3 (from 7.8) did not change the fluorescence signal. Further acidification to pH 6.6 induced moderate signal recovery. Full fluorescence recovery required acidification to pH 5.3.

DISCUSSION

Ion transport in cyanobacteria appears to depend upon a electrochemical H^+-gradient produced across the plasmalemma. In Anacystis nidulans, Paschinger [5] demonstrated an ATP-driven H^+-extrusion across the plasmalemma, which was dependent upon photosynthetic and respiratory energy, presumably ATP synthesis. Raboy and Padan [7] concluded from studies in another cyanobacterium Plectonema boryanum that both ATP-driven and redox driven H^+ pumps are in the plasmalemma.

As regards the mechanism of Na^+ movement, Paschinger [5] suggested that the active Na^+ extrusion was driven by a Na^+/H^+ antiporter system in conjunction with H^+-ATPases; in cells treated with N-N'-dicyclohexylcarbodiimide, Na^+ accumulation was observed, suggesting that the efflux had been ATP driven.

Using the sensitive acridine orange fluorescence quenching technique, it is clear that Na^+/H^+ exchange activity can be demonstrated in Synechococcus 6311 cells. Much larger fluorescence quenching changes are observed after salt-adaptation. The magnitude of the H^+ accumulation (fluorescence quenching) will be dependent on the ratio between Na^+/H^+ exchange and NaCl efflux. The higher permeability to NaCl found in control cells implies that, upon dilution, a significant fraction of intracellular Na^+ will be lost before it can be utilized for H^+ accumulation. In salt-adapted cells the lower NaCl permeability reduces this NaCl loss. An alternative explanation is that an adaptive increase in Na^+/H^+ exchange activity accompanies the development of salt tolerance. Although the extent of fluorescence quenching of acridine orange is highly dependent upon intracellular binding, the large differences between control and salt-adapted cells are not likely to be explained only by differences in intracellular binding since: a) measurements of intracellular buffer capacity, made by titration of cell homogenates from control and salt-adapted cells did not show any significant difference in the pH range between 4 and 8

(data not shown); and b) the increases in protein concentration known to occur in salt adapted cells [10, Blumwald and Packer, in preparation] do not exceed 20 percent of the total protein content of the cell.

In whole cells, it is very difficult to determine the exact mechanism of the Na^+/H^+ exchange. The exchange activity observed might be the expression of the presence of a true antiporter in the plasmalemma. Evidence in support of this suggestion are the Na^+/H^+ stoichiometry and specificity and the effects of various ionphores.

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