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Light-Induced Proton Release by the Cyanobacterium *Anabaena variabilis*[1]

DEPENDENCE ON CO_2 AND Na^+

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ABSTRACT

Light-induced acidification by the cyanobacterium *Anabaena variabilis* is biphasic (a fast phase I and slow phase II) and shown to be sodium-dependent with an optimum concentration of 40 to 60 millimolar Na^+. Cells grown under low CO_2 concentrations at pH 9 (i.e. mainly HCO_3^- present in the medium) exhibited the slow phase II of proton efflux only, while cells grown under low CO_2 concentrations at pH 6.3 (*i.e.* CO_2 and HCO_3^- present) exhibited both phases. Light-induced proton release of phase I was dependent on inorganic carbon available in the bathing medium with an apparent K_m for CO_2 of 20 to 70 micromolar. As was concluded from the CO_2 dependence of acidification measured at different pH of the bathing medium, bicarbonate inhibited phase-I acidification noncompetetively. Acidification was inhibited by acetazolamide, an inhibitor of carbonic anhydrase. Apparently, acidification of phase I is due to a light-dependent uptake of CO_2 being converted to HCO_3^- by a carbonic anhydrase-like function of the HCO_3^--transport system (M Volokita, D Zenvirth, A Kaplan, L Reinhold 1984 Plant Physiol 76: 599–602) before or during entering the cell, thus releasing one proton per CO_2 converted to HCO_3^-.

Aquatic plants are able to actively accumulate DIC[3] (12). Accordingly, cyanobacteria have been shown to effectively take up DIC from the medium, which may result in a several thousandfold accumulation (9, 13). This DIC uptake is strictly light dependent (13, 17) and, as was shown with *Anabaena variabilis*, may be due to a primary electrogenic pump (11). Considerable evidence has accumulated that HCO_3^- can be transported by several cyanobacterial species (*e.g.* 4, 7, 11, 13, 14), but it is controversial whether CO_2 actively enters the cell (1, 27).

Bicarbonate uptake is not a H^+/HCO_3^- symport in *A. variabilis* (29), although high rates of light-induced acidification occur in *Anabaena* and other blue-green algae (for review, see Scherer et al. [25]). In part, this acidification is due to an ATPase localized on the cytoplasmic membrane (8, 23, 24), as well as to light-induced proton release (i.e. phase I) resulting from the conversion of CO_2 to HCO_3^- during the uptake of CO_2 (18). For the latter mechanism data are presented in this paper. Our findings are in favor of the hypothesis that HCO_3^- is the carbon species transported across the plasma membrane of *A. variabilis*, supporting the model of Volokita *et al.* (27).

MATERIALS AND METHODS

Species and Growth. *Anabaena variabilis* (ATCC 29413) was grown either in a medium of high ionic strength according to Arnon et al. (2) or low ionic strength (in BG-11 medium; see Ref. 22) under nitrogen-fixing conditions at 30°C and 13 W/m² (continuous white fluorescent light). The Na^+-concentration in BG-11 medium under these conditions was 0.2 mM. CO_2 supply was 1.5% v/v (high-CO_2 cells) or 0.03% v/v (low-CO_2 cells) as indicated in the figures.

Harvest and Preparation of Cells. Filaments grown in Arnon's medium usually were harvested after 2 d of growth in the late logarithmic phase (5 min, 3000*g*), washed and resuspended in a medium containing 3 mM glycylglycine/NaOH, 75 mM KCl, 75 mM NaCl, 5 mM $MgCl_2$ (pH 6.3), yielding a concentration of about 500 mg Chl per ml; the suspension was kept on ice. This holds for the experiments described in Figures 4 and 5. The storage on ice somewhat decreased photosynthetic oxygen evolution but all activities reported here were found constant during the measurements. For all other experiments, filaments grown in BG-11 medium were withdrawn from the culture vessel and immediately transferred to the reaction chamber.

Determination of Acidification. Cells were suspended in a plexiglass chamber (2.4 ml), equipped with a Clark-type O_2 electrode (YSI), a magnetic stirrer, and a pH-electrode (Radiometer PHM 62, GK 2321C Copenhagen). Reaction temperature was 24°C, samples were illuminated with saturating red light (RG cut-off filter by Schott, Mainz, Germany). The reaction medium was either BG-11 or glycylglycine buffer as mentioned above. Decreased levels of DIC in the reaction medium were obtained by bubbling with CO_2-depleted air for several hours, the air prepared by filtering it through 4 M NaOH and 0.5 M $Ba(OH)_2$ solutions. Chl concentrations during the measurement are given in the legends. After transfer into the reaction chamber, the appropriate pH was adjusted with 50 mM HCl or 50 mM NaOH. After calibration with defined volumes (usually 1 μl) of acid or base, the cells were incubated in the dark until a stable pH trace was obtained (after 6–8 min). Additions were made before calibration. To obtain reproducible results, the incubation time in the dark was kept constant.

RESULTS

Effect of Na^+ and Li^+. The kinetics of proton efflux of *A. variabilis* grown and measured in BG-11 medium in the presence of CO_2 (see legend) is shown in Figure 1. In the absence of Na^+, no acidification was observed. Addition of 46 mM Na^+ led to a biphasic acidification. In Figure 2 the sodium dependence of phase I is shown in detail. Forty to 60 mM was found as the

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[3] Abbreviations: DIC, Dissolved inorganic carbon; ATCC, American Type Culture Collection.

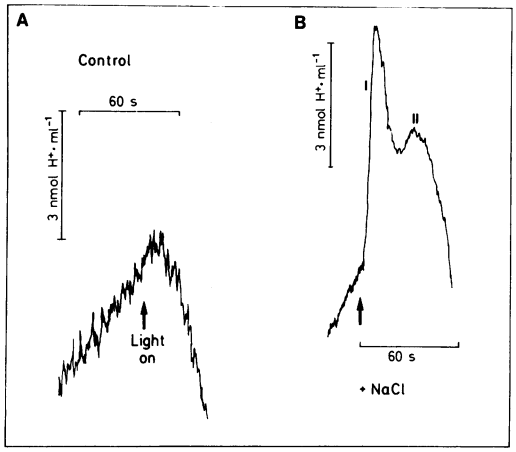

FIG. 1. Light-induced acidification of the medium by *A. variabilis*, grown at pH 6.3 with 1.5% CO_2 (v/v) in BG-11 medium. Cells (equivalent to 8.5 μg Chl × ml^{-1}) were transferred immediately from the growth vessel to the reaction chamber and measured in BG-11 medium in the absence (A) or in the presence (B) of 46 mM NaCl. I, II: phase I and phase II of proton release.

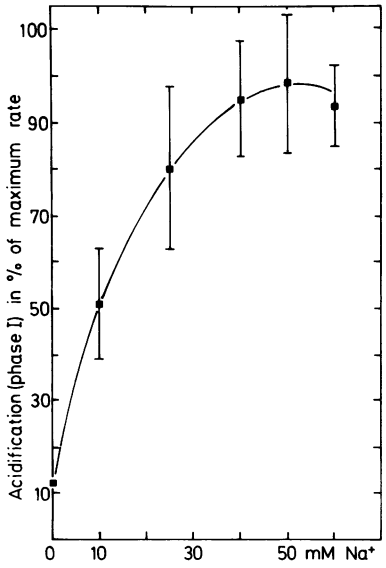

FIG. 2. Influence of NaCl on phase-I acidification of *A. variabilis*. Growth of cells and assay conditions: see legend of Figure 1 and "Materials and Methods;" 100% correspond to 270 to 1100 μmol H^+ × mg Chl^{-1} × h^{-1}. Average values measured with four different culture batches and standard deviation are given. The zero concentration of Na^+ corresponds to 0.2 mM Na^+.

optimum concentration. Cells grown in Arnon's medium, stored on ice, and measured in glycylglycine buffer (see "Materials and Methods") without sodium present also showed an acidification with a much lower rate stimulated again by addition of sodium. Potassium ions did not induce proton efflux whereas lithium ions induced only a weak first phase and then inhibited acidification. Li^+ strongly affected photosynthetic O_2 evolution (Fig. 3) with a maximum inhibition at 50 to 60 mM. Inhibition of O_2 evolution by Li^+ could be partly reversed by Na^+ (data not shown).

Influence of Growth pH. Cells grown at a pH of about 9 under low-CO_2 conditions showed no phase-I acidification (Fig. 4), only the slow phase II of proton efflux was seen with a lag phase of 10 to 20 s before the maximum rate was attained. Growth of *A. variabilis* under low-CO_2 conditions at pH 6.3 led to a phase-I

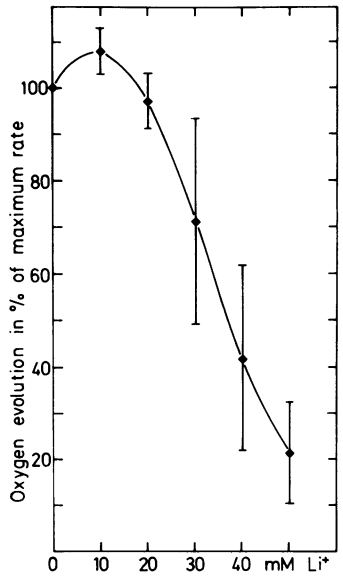

FIG. 3. Inhibition of oxygen evolution by LiCl in the absence of additional Na^+ at saturating DIC. Growth of cells and assay conditions: see legend of Figure 1 and "Materials and Methods;" 100% correspond to 100 to 223 μmol O_2 × mg^{-1} Chl × h^{-1}. Average values and standard deviation are given as obtained from three different culture batches.

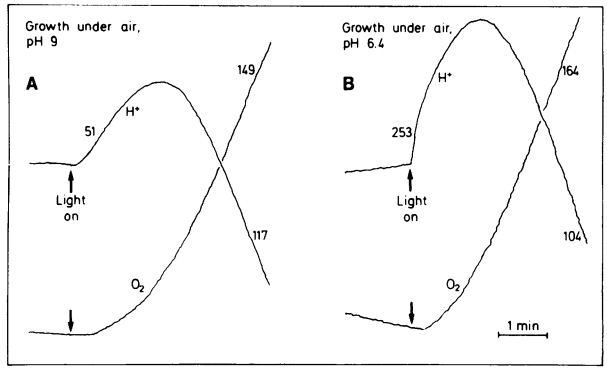

FIG. 4. Light-induced acidification of cells grown for 2 d under air at pH 9.4 (A) and air at pH 6.4 (B) in BG-11 medium. The pH of 6.4 of the culture medium was adjusted by adding 20 mM of 2-(*N*-morpholino)ethanesulfonic acid (MES/NaOH buffer). The assay was performed with 40 mM NaCl present at pH 6.3 and cells equivalent to 12 μg of Chl × ml^{-1}. Figures are given as μmol × mg^{-1} Chl × h^{-1}.

proton efflux with a lag phase of 1 to 3 s (Fig. 4), as was observed with high-CO_2 cells (1.5% CO_2 v/v, pH 6.3).

Dependence on DIC. The phase-I acidification was found to be dependent on inorganic carbon, whereas phase-II proton efflux was not. The influence of CO_2 at different pH of the bathing medium, measured with cells grown at pH 6.3 is shown in Figure 5A. Generally, light-induced acidification was lower at alkaline pH. A kinetic analysis of the data (Fig. 5B) revealed a Michaelis-Menten kinetics in both cases, suggesting a noncompetitive inhibition at alkaline pH. The apparent K_m for CO_2 was found variable between 20 and 70 μM in different batches of *Anabaena*. It was also dependent on the treatment of the cells preceding measurement (data not shown).

Inhibitor of Carbonic Anhydrase. Acetazolamide is known as an inhibitor of the enzyme (19) and substantially inhibited phase I of light-induced acidification (Table I). Phase II, as measured with air-grown cells, was not inhibited by acetazolamide (Table I).

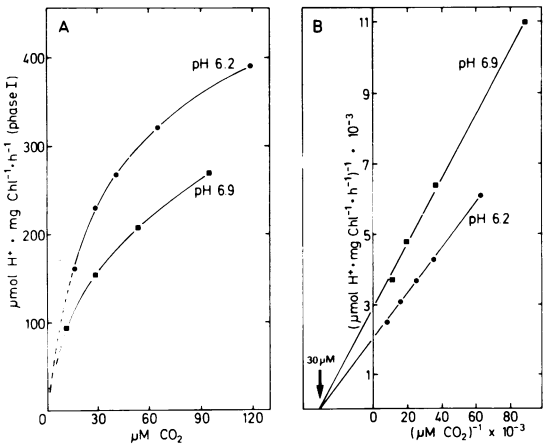

FIG. 5. Dependence of phase I acidification on DIC of *A. variabilis*. Cells grown for 2 d under 1.5% CO_2 (v/v) in a medium according to Arnon *et al.* (2) were measured in glycylglycine buffer. A CO_2 free buffer was prepared as described in "Materials and Methods"; Chl was 30 µg × ml^{-1}. (A) Dotted lines indicate that the initial amount of CO_2 in the buffer has been estimated only. After addition of DIC, as $NaHCO_3$, the pH was adjusted with HCl and the light switched on after 2 to 4 min of equilibration. The pH-dependent CO_2 content was calculated from total inorganic carbon according to Buch (6). Data of a typical experiment are given. (B) A double-reciprocal plot of the data of (A).

DISCUSSION

Light-induced acidification in *A. variabilis* is biphasic. The second, slow phase is most likely due to a proton-translocating ATPase localized in the cytoplasmic membrane (8, 23, 24), while the function of the first, fast phase of acidification is not yet known. Despite considerable efforts, the mechanism of DIC uptake in cyanobacteria is still unclear, but there is wide agreement that HCO_3^- can pass the cytoplasmic membrane (4, 7, 11, 13, 14). Whether CO_2 can pass actively the membrane of *A. variabilis* is disputed (1, 27). In this paper, we present evidence based on proton flux measurements that CO_2 is converted to HCO_3^- before or during it enters the cell.

It has been shown by many investigators that photosynthesis (15) and DIC uptake (10, 16, 21) depend on sodium ions. In *Anabaena*, grown under high CO_2-concentrations, photosynthetic O_2 evolution is slightly stimulated by Na^+ (data not shown) and strongly inhibited by LiCl (Fig. 3). It may be possible, although we have not checked it, that the stimulatory effect of Na^+ an O_2 evolution is dependent on the DIC concentration during growth. Miller and Canvin (16) reported that O_2 evolution and HCO_3^- uptake of *Synechococcus* is inhibited by Li^+ under DIC limiting conditions. Our data confirm their finding that inhibition of O_2 evolution by Li^+ can be overcome by high Na^+ concentrations. Since light-induced acidification depends on sodium ions (Figs. 1 and 2) with a similar optimum concentration as reported for its function in DIC uptake of *Anabaena* M-3 (10) we conclude that light-induced acidification apparently is connected with DIC uptake. Although a Na^+/HCO_3^--symport may be possible (21), the function of sodium in DIC transport is not yet clear and further investigations on Na^+ fluxes are necessary, because sodium ions also may be important in osmoregulation (5, 20). A light-dependent uptake of $^{22}Na^+$ in *Anabaena* has been shown recently (S Scherer, unpublished results).

A DIC-depleted reaction medium allowed us to determine the influence of DIC on phase-I acidification (Fig. 5A). The rate of proton release of pH 6.9 was significantly lower than at pH 6.3; above pH 8 no acidification was found at all. This does not imply that no CO_2 is taken up at this pH; CO_2 uptake above pH 8 was strongly limited by the CO_2 concentration in our closed system, which was approximately 5 to 10 µM. The acidification expected is too small to be measured by our pH-electrode. Assuming that CO_2 and not HCO_3^- is responsible for the proton release observed, a K_m between 20 and 70 µM could be determined, which is in the same order of magnitude as the K_m found for DIC uptake in *Anabaena* (9, 27). At pH 6.9, as compared to pH 6.3, a larger fraction of DIC is present as HCO_3^-, which, according to the data of Volokita *et al.* (27), noncompetitively inhibits the use of CO_2 as a carbon source of *A. variabilis*. Although (in Ref. 27) these authors refer to a competitive inhibition in the text, their Dixon plot clearly exhibits a noncompetitive type. Conclusively, phase-I acidification should be dependent on CO_2. Additional support for this hypothesis arises from the experiment shown in Figure 4: cells grown under air at pH 9.4 (with a high HCO_3^-/CO_2 ratio) showed no phase I, but cells grown under air at pH 6.3 (with approximately 55% of DIC present in the form of CO_2) clearly exhibited phase I.

The data reported in this study on light-induced phase-I acidification in *A. variabilis* are in excellent accordance with one of the models for carbon uptake published for *A. variabilis* M-3 (27), which is shown in a slightly modified form in Figure 6. In this model, a carbonic anhydrase-like function is suggested to be closely associated with the HCO_3^- porter. CO_2 is converted to HCO_3^- before it is transported across the cytoplasmic membrane, thus liberating one proton per CO_2, which is measured as phase-I acidification in our experiments. Recently, Ogawa and Kaplan (18) reported on a molar CO_2:H^+-stoichiometry of 1:1 in a mutant of *Anacystis*, again supporting the model of Figure 6. *Anabaena* does not show extracellular carbonic anhydrase activity (26, 28), but the uptake of CO_2 (not HCO_3^-) is inhibited by the carbonic-anhydrase inhibitor ethoxyzolamide (27). The hypothesis is corroborated by the finding that phase-I but not phase-II acidification is inhibited by acetazolamide (Table I). Additional support for the model of Figure 6 emerges from light dependence. Phase I is driven by red light (707 nm) showing an action spectrum (24), which is somewhat similar to that for car-

Table I. *Inhibition of Acidification by Acetazolamide*

Data are given as % of control without inhibitor; 100% correspond to 350 to 712 (phase I), 51 to 75 (phase II), 64 to 132 (alkalization), 187 to 330 (O_2 evolution of CO_2-grown cells), and 95 to 113 (O_2 evolution of air-grown cells) µmol × mg^{-1} Chl × h^{-1}. Average values + from 10 experiments (CO_2-grown cells) are given. DIC concentration was 0.5 to 1 mM. Cells were grown in Arnon's medium (see "Materials and Methods").

Conditions	Proton Efflux		Alkalization	Oxygen Evolution
	Phase I	Phase II		
Control	100	100	100	100
1 mM Acetazolamide				
CO_2-grown cells	42 ± 10	ND[a]	68 ± 18	75 ± 4
Air-grown cells[b]		111 ± 9	108 ± 8	78 ± 19

[a] Not determined. [b] Air-grown cells exhibited no phase I when grown at pH 9.

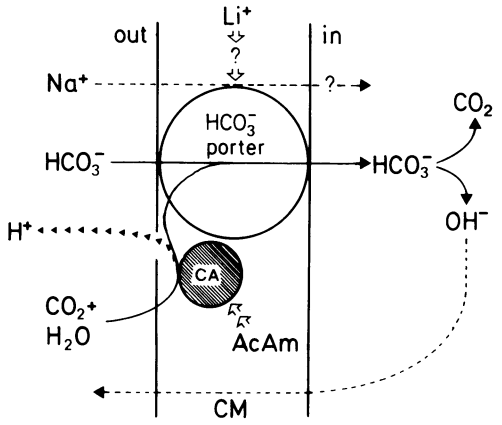

FIG. 6. A possible model for DIC transport across the cytoplasmic membrane, based on Volokita *et al.* (27) and the results presented in this paper. The biochemical function of Na^+ and Li^+ is speculative, although a light-dependent $^{22}Na^+$ uptake with *Anacystis* E_1 and *R. variabilis* could be demonstrated recently in our laboratory (A Kaplan, S Scherer, unpublished data). CA, carbonic-anhydrase like function of the CO_2/HCO_3^- transport system; AcAm, acetazolamide; CM, cytoplasmic membrane; ⇛, presumed inhibitory site.

bon uptake in *Anabaena* M-2 (17).

Aizawa and Miyachi (1) questioned the model for CO_2 transport suggested by Volokita *et al.* (27), assuming that, in addition to a HCO_3^- transport, in *Anabaena* a CO_2 porter is responsible for the uptake of CO_2, transporting CO_2 and not HCO_3^- across the cytoplasmic membrane. If this were true, no protons should be liberated during DIC uptake, which is in contrast to the data on acidification presented in this paper for *Anabaena* and reported most recently for *Anacystis* (18). Of course, DIC uptake mechanisms in cyanobacteria may vary between species (1) and, as expressed by Badger and Andrews (3), "the mechanistic basis for the inorganic carbon concentrating system may not be a simple HCO_3^- pump."

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