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Inorganic Carbon Accumulation Stimulates Linear Electron Flow to Artificial Electron Acceptors of Photosystem I in Air-Grown Cells of the Cyanobacterium *Synechococcus* UTEX 625[1]

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The effect of inorganic carbon (C_i) transport and accumulation on photosynthetic electron transport was studied in air-grown cells of the cyanobacterium *Synechococcus* UTEX 625. When the cells were depleted of C_i, linear photosynthetic electron flow was almost completely inhibited in the presence of the photosystem I (PSI) acceptor *N,N*-dimethyl-*p*-nitrosoaniline (PNDA). The addition of C_i to these cells, in which CO_2 fixation was inhibited with glycolaldehyde, greatly stimulated linear electron flow and resulted in increased levels of photochemical quenching and O_2 evolution. In aerobic conditions substantial quenching resulted from methyl viologen (MV) addition and further quenching was not observed upon the addition of C_i. In anaerobic conditions MV addition did not result in quenching until C_i was added. Intracellular C_i pools were formed when MV was present in aerobic or anaerobic conditions or PNDA was present in aerobic conditions. There was no inhibitory effect of C_i depletion on electron flow to 2,6-dimethylbenzoquinone and oxidized diaminodurene, which accept electrons from photosystem II. The degree of stimulation of PNDA-dependent O_2 evolution varied with the C_i concentration. The extracellular C_i concentration required for a half-maximum rate ($K_{1/2}$) was 3.8 μM and the intracellular $K_{1/2}$ was 1.4 mM for the stimulation of PNDA reduction. These values agreed closely with the $K_{1/2}$ values of extracellular and intracellular C_i for O_2 photoreduction. Linear electron flow to artificial electron acceptors of PSI was enhanced by intracellular C_i, which appeared to exert an effect on PSI or on the intersystem electron transport chain.

O_2 photoreduction in *Synechococcus* UTEX 625 is stimulated by the formation of the intracellular C_i pool (Miller et al., 1988a; Canvin et al., 1990; Li and Canvin, 1997a). When CO_2 fixation is inhibited with iodoacetamide or GLY (Miller and Canvin, 1989) O_2 photoreduction is increased 4- to 5-fold upon the addition of C_i (Miller et al., 1988a; Li and Canvin, 1997a), and under such conditions the rate of O_2 photoreduction approaches the maximum rate of CO_2 fixation (Miller et al., 1988a; Li and Canvin, 1997a).

O_2 photoreduction also results in photochemical quenching of Chl *a* fluorescence, indicating that electron flow in the photosynthetic electron transport chain is stimulated (Miller et al., 1988a, 1991; Badger and Schreiber, 1993; Li and Canvin, 1997a, 1997b). The magnitude of quenching is highly correlated with the size of the intracellular C_i pool (Miller et al., 1991; Crotty et al., 1994) and with the rate of O_2 photoreduction (Li and Canvin, 1997b) in cells in which CO_2 fixation has been inhibited. In previous work, quenching of Chl *a* fluorescence was used as an indicator of O_2 photoreduction (Miller et al., 1991; Badger and Schreiber, 1993).

The electrons required for O_2 photoreduction are derived from the photooxidation of water by PSII, but the actual reduction of O_2 is thought to occur via reduced Fd (Canvin et al., 1990). How the intracellular C_i pool stimulates electron flow in the photosynthetic electron transport chain is unknown.

It was suggested (Miller et al., 1991) that accumulated HCO_3^- may stimulate electron transfer between Q_A^- and plastoquinone (Blubaugh and Govindjee, 1988; Cao and Govindjee, 1988), but the intracellular C_i concentration of 15 to 20 mM required for maximum stimulation of electron transport (Li and Canvin, 1997b) is greatly in excess of that required for the Q_A^--to-plastoquinone transfer (Blubaugh and Govindjee, 1988). Badger and Schreiber (1993) did not observe any effect of added C_i on quenching due to the addition of artificial electron acceptors of PSII or PSI and suggested that HCO_3^- may have a direct effect on reactions involved in O_2 photoreduction. Further study, however, showed that NO_2^- reduction was also stimulated by the addition of C_i (Mir et al., 1995a) and O_2 photoreduction was suppressed by NO_2^- assimilation (Mir et al., 1995b). NO_2^- reduction occurs via reduced Fd (Guerrero and Lara, 1987), and the apparent competition between O_2 photoreduction and NO_2^- reduction suggests that C_i was acting on some component of the electron transport chain.

In view of these findings, we investigated the effect of C_i on the transfer of electrons to artificial electron acceptors under a range of conditions. At saturating light the reduction of PSI acceptors such as PNDA and MV was stimulated by the formation of the intracellular C_i pool, but no effect of C_i addition on the reduction of PSII acceptors such

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Abbreviations: BTP, 1,3-bis[Tris(hydroxymethyl)-methylamino] propane; CA, carbonic anhydrase (carbonate dehydratase, EC 4.2.1.1); Chl, chlorophyll; C_i, dissolved inorganic carbon (CO_2 + HCO_3^- + CO_3^{2-}); DADox, oxidized diaminodurene; DMQ, 2,6-dimethylbenzoquinone; *F*, Chl fluorescence measured at any time in the absence of a saturating flash; F^*_m, maximum fluorescence in the absence of C_i; GLY, glycolaldehyde; GOD, Glc oxidase system; $K_{1/2}$, concentration required for half-maximum rate; MV, methyl viologen; PNDA, *N,N*-dimethyl-*p*-nitrosoaniline.

as DMQ and DADox was observed. These results suggest that the intracellular C_i pool exerts an effect on electron transfer in PSI or on the intersystem electron transport chain.

MATERIALS AND METHODS

Organism and Culture Conditions

The unicellular cyanobacterium *Synechococcus* UTEX 625 (University of Texas Culture Collection, Austin) was grown at 30°C in modified, unbuffered Allen's medium bubbled with air (0.036% [v/v] CO_2) as previously described (Espie and Canvin, 1987). The cultures were illuminated at a light intensity of 80 μmol m^{-2} s^{-1}. Cells were harvested about 48 h after incubation as described previously (Li and Canvin, 1997a). At that time the Chl *a* concentration was between 5 and 7 μg mL^{-1}, the C_i was about 50 μM, and the pH was about 10.6 in the medium.

Experimental Conditions

Cells were harvested and washed three times in BTP-HCl (25 mM, pH 8.0) by centrifugation for 45 s at 10,000*g* in a microfuge at room temperature. Cells were resuspended at 5 to 8 μg Chl mL^{-1} in the same buffer, which had been previously bubbled with N_2 to remove most of the C_i (Miller et al., 1984). The resuspended cells were placed in a glass chamber at 30°C and 80 μmol m^{-2} s^{-1} light in the presence of 25 mM NaCl and 25 μg mL^{-1} CA and allowed to fix contaminant C_i in the buffer. They were subsequently used for the various experiments. The Chl concentration was determined spectrophotometrically at 665 nm after extraction in methanol (MacKinney, 1941).

The simultaneous measurement of O_2 evolution and fluorescence yield was performed at 30°C in an O_2 electrode apparatus (model DW2, Hansatech Instruments, King's Lynn, UK). Two milliliters of the cell suspension was transferred and allowed to reach the CO_2 compensation point, as monitored by fluorescence yield and O_2 evolution (Miller et al., 1988b). The suspension was stirred with a rotating magnetic bar, and chemicals were introduced into the reaction chamber through a small port. The fluorescence yield was monitored with a pulse amplitude modulation fluorometer (PAM-101, Walz, Effeltrich, Germany) as described by Schreiber et al. (1986). Cell suspensions were illuminated by a tungsten-halogen projector lamp at a light intensity of 80 μmol m^{-2} s^{-1}. The pulse measuring beam (100 kHz) was 1 μmol m^{-2} s^{-1}. The oxidation state of Q_A was routinely measured at 60-s intervals with a 1-s flash of high-intensity white light at 1600 μmol m^{-2} s^{-1}. The results were recorded on a Linear recorder. When CO_2 fixation was to be inhibited, GLY was added to a final concentration of 15 mM. When required, O_2 was removed from the solution by the addition of 10 mM Glc, 100 μg mL^{-1} GOD, and 50 μg mL^{-1} catalase (Miller et al., 1991).

The simultaneous measurement of CO_2 uptake, O_2 evolution, and O_2 photoreduction was performed by MS as previously described (Miller et al., 1988a; Li and Canvin, 1997a). Six milliliters of the cell suspension was transferred to the reaction chamber (20 mm in diameter), which was connected via a membrane inlet to the mass spectrometer. The experiments were performed in the presence of CA so that the CO_2 concentration was always directly proportional to the C_i concentration (Badger and Price, 1994). To distinguish the CO_2 leaking from the cells due to C_i accumulation from the respiratory CO_2, we used ^{13}C-labeled C_i in all experiments. The short time of incubation precluded entry of ^{13}C into respiratory substrates, and $^{13}CO_2$ evolution was not observed during the dark at the end of the experiment. Rates of O_2 evolution and uptake were calculated from the slopes of the 32 and 36 m/z signals after appropriate corrections for leak rates (2% h^{-1}) and changes in the mole fractions of $^{16}O_2$ and $^{18}O_2$ during the course of the experiment were made (Miller et al., 1988a; Li and Canvin, 1997a). The rates of O_2 evolution and uptake were corrected for the rates of O_2 evolution and uptake that occurred in C_i-depleted cells in the light in the absence of any other addition (20–30 μmol mg^{-1} Chl h^{-1}). The rates of CO_2 fixation were calculated from the slope of the 45 m/z signal (Li and Canvin, 1997b).

MS

Concentrations of dissolved $^{16}O_2$, $^{18}O_2$, $^{12}CO_2$, and $^{13}CO_2$ (m/z 32, 36, 44, and 45, respectively), in the 6-mL reaction chamber were measured simultaneously with a mass spectrometer (model MM 14–80 SC, VG Gas Analysis, Middlewich, UK) equipped with a membrane inlet system. The mass spectrometer was controlled using software (Spectrascan 5.3.1 and Petrasoft 5.3.2, VG Gas Analysis), and the output signal was directed to a 486 computer (Altair, Kingston, Canada). Calibration for CO_2 was performed by injecting known amounts of $KH^{13}CO_3$ into the stoppered cuvette in 6 mL of 25 mM BTP-HCl, pH 8.0, and calculating the equilibrium concentration of CO_2 at 30°C, as described by Miller et al. (1988c). Calibrations for $^{16}O_2$ and $^{18}O_2$ were performed as previously described (Mir et al., 1995b; Li and Canvin, 1997a).

Chl *a* Fluorescence

For illuminated *Synechococcus* UTEX 625 cells, F^*_m, which was very close to that seen after the addition of DCMU, was achieved after illumination with actinic light in the complete absence of C_i. The fluorescence terminology used in this paper conforms as closely as possible to the recommended standard nomenclature (Van Kooten and Snel, 1990), with a few additional terms described in more detail by Miller et al. (1991). The parameter $(F^*_m - F)/F^*_m$ was calculated and used as an indicator of the rate of linear electron flow.

Intracellular C_i Pool Size

The size of the intracellular C_i pool was measured with the mass spectrometer under inhibited conditions of CO_2 fixation. Since the experiments were performed in the presence of CA in the reaction mixture, the CO_2 concentration would always be directly proportional to the C_i concentration. In the presence of GLY the C_i transported into the cells

was not fixed and leaked back into the medium when the lights were turned off. The intracellular C_i was determined as the difference between the external $[C_i]$ in the light and the $[C_i]$ that occurred upon darkening the cells. The internal $[C_i]$ was calculated using an intracellular volume of 75 μL mg^{-1} Chl (Miller et al., 1988c).

Measurement of Photoreduction of PNDA

PNDA photoreduction was determined spectrophotometrically using an extinction coefficient of 3.4×10^4 at 440 nm.

Chemicals

PNDA, MV, DMQ, DADox, GLY, CA, and BTP were obtained from Sigma. PNDA, DMQ, and DADox were dissolved in DMSO, and DCMU was dissolved in methanol. $K_2{}^{13}CO_3$ (99 atom% ^{13}C) was obtained from MSD Isotopes (Montreal, Quebec, Canada). The $KH^{13}CO_3$ was dissolved in BTP-HCl, pH 8.0, as a concentrated solution for addition to the reaction medium (Miller et al., 1988b). $^{18}O_2$ (97.4 atom% ^{18}O) was supplied by Cambridge Isotope Laboratories (Andover, MA).

RESULTS

Effect of PNDA on CO_2 Fixation and O_2 Photoreduction

Previous work on O_2 photoreduction, NO_2^- reduction, and CO_2 fixation in cyanobacteria showed that the competition or interaction among the reduction of these species varied as a function of light intensity (Mir et al., 1995b). The effect of PNDA concentration and light intensity were explored to determine the interaction of PNDA with O_2 photoreduction and CO_2 fixation.

O_2 photoreduction in GLY-inhibited cells was very sensitive to PNDA addition and was totally inhibited by 200 μM PNDA (Fig. 1). CO_2 fixation in noninhibited cells was less sensitive to PNDA addition and was inhibited only by about 80% at the highest PNDA concentration tested.

The complete inhibition of O_2 photoreduction by 200 μM PNDA did not change over the range of light intensity investigated whether or not CO_2 fixation was inhibited (Fig. 2). However, the inhibition of CO_2 fixation by 200 μM PNDA did change with light intensity. At low light intensity (80 μmol m^{-2} s^{-1}), CO_2 fixation was inhibited 100%, but as the light intensity was increased the inhibition decreased to about 50% (Fig. 2, A and C).

When CO_2 fixation was not inhibited (Fig. 2A), the addition of PNDA as another electron acceptor did not increase the rate of O_2 evolution, even at the highest light intensity used. When CO_2 fixation was inhibited, however, O_2 evolution with only O_2 as the electron acceptor (Fig. 2B, −PNDA) was only 50% of that in noninhibited cells (compare EO_2 − PNDA, Fig. 2A, with EO_2 − PNDA, Fig. 2B). The added electron-accepting capacity of PNDA restored O_2 evolution in inhibited cells (Fig. 2B) to the rates observed in noninhibited cells (Fig. 2A). Cyanobacterial cells

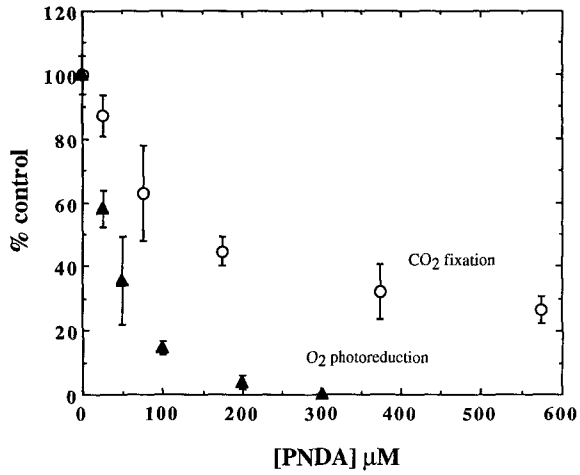

Figure 1. The effect of PNDA on photosynthetic CO_2 fixation and O_2 photoreduction at a light intensity of 400 μmol m^{-2} s^{-1}. Rates of CO_2 fixation and O_2 photoreduction were measured as described in "Materials and Methods." The rate of CO_2 fixation in the absence of PNDA was 254 μmol mg^{-1} Chl h^{-1} with 200 μM C_i, and the inhibitory effect of PNDA on CO_2 fixation (○) was measured after successive additions of PNDA to yield increasingly higher concentrations. The control rate of O_2 photoreduction was 168 μmol mg^{-1} Chl h^{-1} (▲) and was measured after the addition of 100 μM C_i in the light in the presence of 15 mM GLY. PNDA in increasing concentrations was then added to inhibit O_2 photoreduction. The [Chl] was 5.5 μg mL^{-1}. The data shown are means ± SD (n = 3).

in the light can deplete the $[C_i]$ in the medium to 0 levels. If CO_2 fixation is inhibited by GLY or the cells are placed in the dark, however, C_i soon appears in the medium. C_i can, of course, also be added with the addition of experimental chemicals.

Given that 1.0 μM C_i in the extracellular medium will greatly stimulate O_2 photoreduction (Li and Canvin, 1997b), it is important that C_i not be present upon the addition of PNDA when investigating the effect of C_i on PNDA reduction. Because CO_2 fixation was not totally inhibited by PNDA addition (Figs. 1 and 2), any C_i that was added with PNDA could be fixed if the PNDA was added before the GLY. An experiment was then conducted in which PNDA was added before or after GLY (Fig. 3). The results were also affected by light intensity (Fig. 3); at high light intensity PNDA reduction was stimulated 4-fold by the addition of C_i when GLY was added after PNDA, but a much smaller stimulation was observed when GLY was added before the PNDA (Fig. 3B). At a low light intensity, little stimulation (20%) of PNDA reduction by C_i was observed when PNDA was added after GLY, whereas 70% stimulation was observed if PNDA was added before GLY. Clearly, to see the maximum effect of C_i on PNDA reduction, it is advantageous to use high light and to add the PNDA before GLY. Therefore, when measuring O_2 evolution due to PNDA addition, we subsequently adopted that protocol. However, that protocol was not possible when measuring fluorescence when the light intensity was lower (80 μmol m^{-2} s^{-1}) and GLY was added before PNDA.

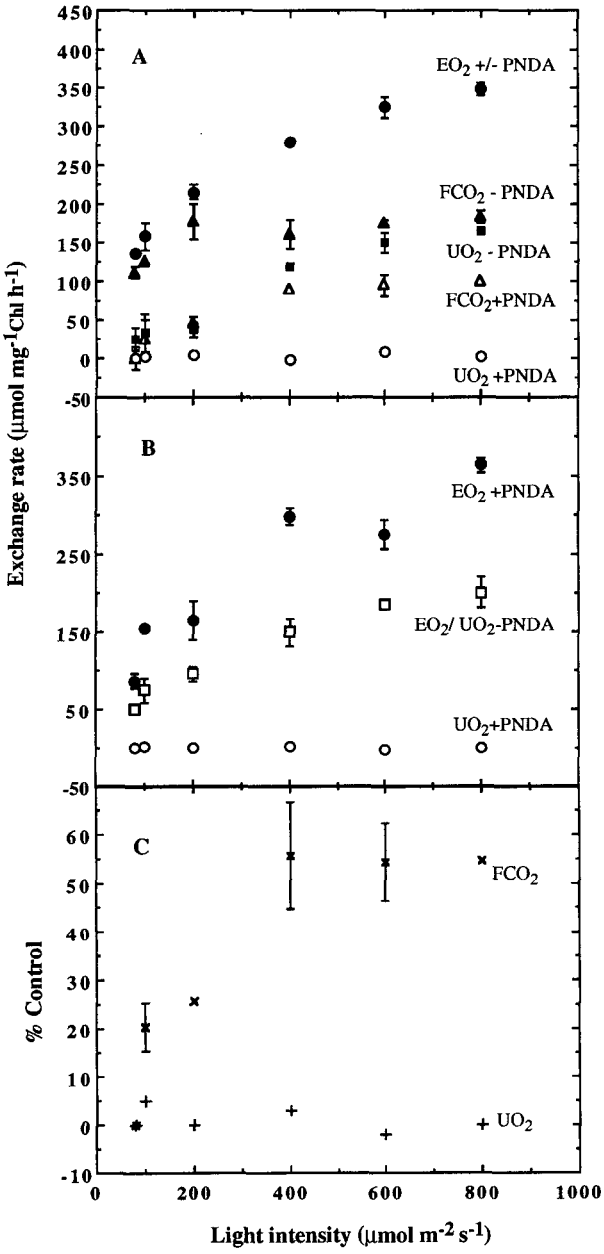

Figure 2. The relationship between light intensity and the effect of PNDA (200 μM) addition on gas exchange of noninhibited cells (A), on gas exchange in which CO_2 fixation was inhibited (B), and on the percentage of CO_2 fixation or O_2 photoreduction (from A) remaining after PNDA addition (C). In A, gross O_2 evolution (●, EO_2), CO_2 fixation (△, ▲, FCO_2), and O_2 photoreduction (○, ■, UO_2) were measured before (−PNDA) and after (+PNDA) PNDA addition. In B, O_2 photoreduction (○, □, UO_2) and evolution (●, □, EO_2) in the presence of 15 mM GLY (added before C_i) were measured before (−PNDA) and after (+PNDA) addition. In C, the remaining percentage of the rates of CO_2 fixation (×, FCO_2) and O_2 photoreduction (+, UO_2) in the presence of PNDA are shown. The O_2 concentration was 240 μM. The C_i was added in the light at 200 μM (A) or 100 μM (B). The rates of CO_2 fixation and O_2 exchange were measured by MS and calculated as described in "Materials and Methods." The [Chl] was 5.3 μg mL^{-1}. The data shown are means ± SD (n = 3).

C_i Effect on PNDA and MV-Induced Quenching

PNDA and MV accept electrons from PSI (Elstner and Zeller, 1978; Fujii et al., 1990), and the abilities of PNDA and MV to act as electron acceptors in the absence of C_i were examined. The experiments were carried out in the presence (Figs. 4A and 5A) or absence (Figs. 4B and 5B) of O_2 under conditions in which CO_2 fixation was inhibited with GLY (Miller and Canvin, 1989)

Figure 4 shows the effect of C_i on PNDA-induced quenching of Chl *a* fluorescence under aerobic and anaerobic conditions. The rate of O_2 evolution due to PNDA photoreduction was also measured (Fig. 4A). In the absence of C_i, at the CO_2 compensation point the addition of 200 μM PNDA initiated quenching of Chl *a* fluorescence and O_2 evolution simultaneously (Fig. 4A). Quenching also occurred in the absence of C_i under anaerobic conditions (Fig. 4B). The addition of C_i resulted in a 40% increase in

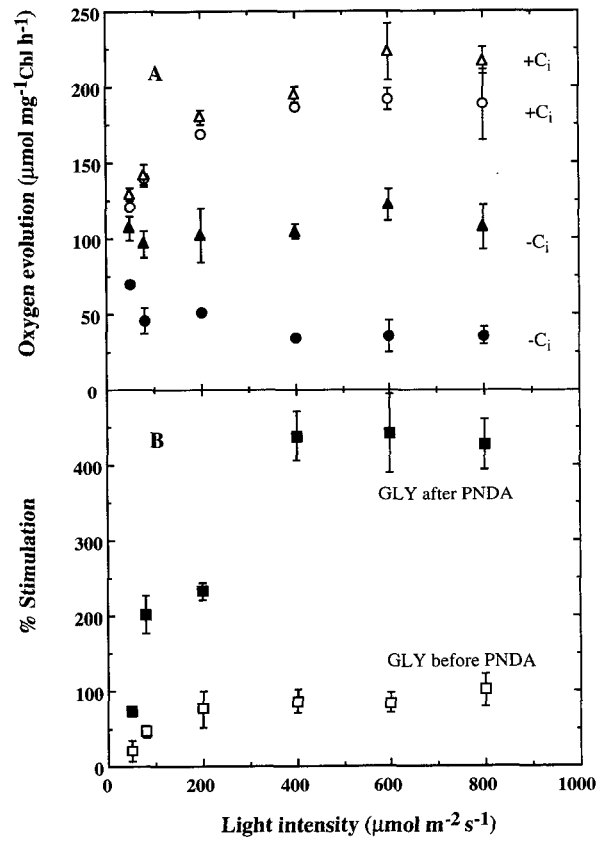

Figure 3. The effect of light intensity on O_2 evolution (A) and the percentage stimulation of O_2 evolution by the addition of C_i (B) with PNDA as the electron acceptor. Cells were incubated in the light to reach the CO_2 compensation point, and O_2 evolution due to PNDA reduction was measured using the O_2 electrode. The reactions were started by the addition of 200 μM PNDA, followed by 200 μM C_i. The initial O_2 was 80 μM. O_2 evolution was calculated as described in "Materials and Methods." CO_2 fixation was inhibited with 15 mM GLY either 2 min before (△, ▲) or 5 min after (○, ●) the addition of PNDA (A). The stimulation of PNDA reduction by C_i (B), in which GLY was added before (□) or after (■) PNDA is shown. The data shown are means ± SD for three separate measurements with the same cells (5.5 μg Chl mL^{-1}).

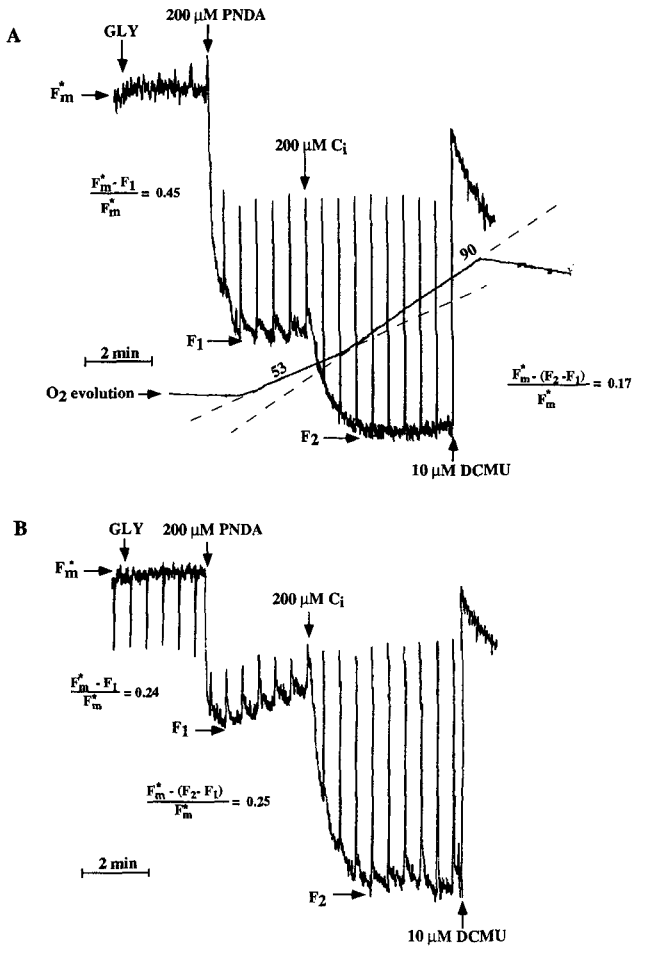

Figure 4. The effect of C_i on PNDA-dependent quenching of Chl *a* fluorescence and O_2 evolution under aerobic (A) and anaerobic (B) conditions in cells of *Synechococcus* UTEX 625. The cells were preincubated in the light to remove C_i from the medium in the presence of 25 mM NaCl and 25 μg mL^{-1} CA. GLY was added to a concentration of 15 mM. The reaction was started by the addition of 200 μM PNDA and followed by the addition of 200 μM C_i as shown. Actinic light was 80 μmol m^{-2} s^{-1}. The initial O_2 concentration was 150 μM and O_2 evolution was recorded using an O_2 electrode (A). Rates of O_2 evolution are given as μmol mg^{-1} Chl h^{-1} (the numbers next to the trace). When required, O_2 was removed from the reaction mixture (B) by the addition of GOD. [Chl] was 6.6 μg mL^{-1}. DCMU was added to a final concentration of 10 μM.

quenching under aerobic conditions (Fig. 4A) and a 100% increase in quenching under anaerobic conditions (Fig. 4B). The increased quenching induced by the addition of C_i was transiently relieved when cells were illuminated with a saturating flash of light, indicating that the quenching caused by the addition of C_i was largely photochemical quenching (Fig. 4). When O_2 evolution was measured (Fig. 4A), the addition of C_i stimulated O_2 evolution about 2-fold. When DCMU was added to the medium, the fluorescence yield rapidly returned to a level close to the original level (F^*_m), demonstrating that most of the quenching induced was photochemical.

The addition of 1 mM MV to GLY-inhibited cells caused strong photochemical quenching of Chl *a* fluorescence in the presence of O_2 (Fig. 5A). Quenching was maximal even in the absence of C_i (Fig. 5A), and the calculated electron flow rate was equivalent to the rate when both CO_2 and O_2 were possible electron acceptors (Li and Canvin, 1997a). Under aerobic conditions the photoreduction of MV, as determined from O_2 uptake with MS, proceeded at a high and constant rate of 200 μmol mg^{-1} Chl h^{-1} even in the absence of C_i (data not shown). The addition of C_i caused no stimulatory effect on fluorescence quenching, and quenching was inhibited by the addition of DCMU (Fig. 5A). Thus, MV can act as an efficient electron acceptor in the absence of C_i under aerobic conditions.

When the same experiment with MV was performed in anaerobic conditions different results were observed (Fig.

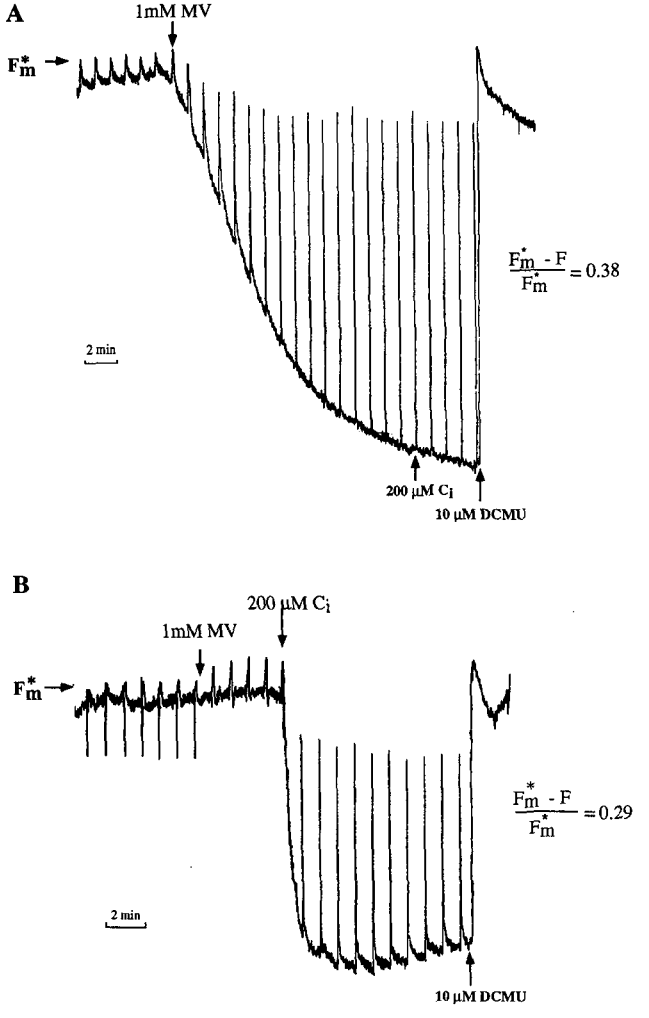

Figure 5. MV-dependent quenching of Chl *a* fluorescence in air-grown cells of *Synechococcus* UTEX 625 in the presence (A) and absence (B) of O_2. Cell suspensions (5.1 μg Chl mL^{-1}) were incubated as described in "Materials and Methods" and allowed to reach the CO_2 compensation point. Photosynthesis was inhibited by the addition of 15 mM GLY. O_2 was removed from the medium (B) by the addition of the GOD. The initial O_2 concentration was 150 μM. Actinic light was 80 μmol m^{-2} s^{-1}. The reaction was started by the addition of 1 mM MV. At the times indicated, 200 μM C_i was added. DCMU was added to a final concentration of 10 μM.

5B); cells of *Synechococcus* UTEX 625 exhibited maximum fluorescence in the absence of C_i, and when illuminated with a saturating flash of light some apparent oxidation of Q_A occurred. The addition of MV in the presence of GLY did not cause any quenching of Chl *a* fluorescence, but increased fluorescence was observed with a saturating flash of light. The addition of C_i now resulted in significant fluorescence quenching (Fig. 5B). The quenching was maximal immediately after the addition of C_i and the electron flow was 76% of that previously calculated in aerobic conditions. Most of the quenching induced by the addition of C_i was transiently relieved when cells were illuminated with a saturating flash of light, indicating that the quenching caused by the addition of C_i was due to increased oxidation of Q_A. When DCMU was added to the medium, the fluorescence yield rapidly returned to the original level, further demonstrating that the quenching was photochemical (Krause et al., 1982).

Suppression of Photoreduction of PNDA in the Absence of C_i and C_i Accumulation during Photoreduction of PNDA and MV

To obtain good fluorescence measurements it was necessary to use a light intensity that was subsaturating for electron transport (Li and Canvin, 1997a). It was also necessary to add GLY to inhibit CO_2 fixation before the addition of the artificial electron acceptors, creating the possibility that some C_i was added to the reaction mixture with the artificial electron acceptors or was produced by metabolism of the cells. Nevertheless, even with those conditions it was possible to show that the addition of C_i stimulated electron transport to PNDA (Fig. 4) and MV (Fig. 5).

O_2 photoreduction is only saturated at light intensities greater than 500 μmol m^{-2} s^{-1} (Li and Canvin, 1997a), and any C_i addition with the artificial electron acceptors could be fixed by photosynthesis if the artificial electron acceptor was added before the CO_2 fixation inhibitor (note that CO_2 fixation is not completely inhibited by the addition of PNDA; Fig. 2). With these conditions fluorescence cannot be measured, but simultaneous O_2 evolution and C_i measurements can be made in the mass spectrometer when artificial electron acceptors are added to the cells.

The results of an experiment with saturating light and PNDA addition before GLY are shown in Figure 6. The addition of PNDA to cells at the CO_2 compensation point resulted in a small amount of O_2 evolution, which ceased after about 4 min. After GLY addition the addition of C_i resulted in a pronounced O_2 evolution rate (167 μmol mg^{-1} Chl h^{-1}; Fig. 6).

The rate of photoreduction of PNDA was also determined from the decrease in A_{440}. A higher rate of photoreduction of PNDA (208 μmol mg^{-1} Chl h^{-1}) was observed in the presence of C_i compared with the rate (5 μmol mg^{-1} Chl h^{-1}) in its absence. The pronounced O_2 evolution was not due to CO_2 fixation. The C_i taken up by the cells was quantitatively released back into the medium when the actinic light was turned off (Fig. 6). The calculated intracellular C_i pool was 19 mM, which is smaller than the pool

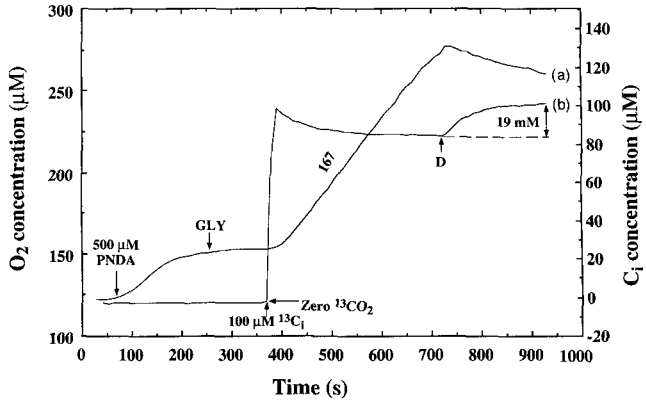

Figure 6. C_i effect on PNDA-dependent O_2 evolution (a) and C_i accumulation (b) monitored by MS with air-grown cells of *Synechococcus* UTEX 625. The cells were preincubated in the light to remove C_i from the medium in the presence of 25 mM NaCl and 25 μg mL^{-1} CA. The reaction was started by the addition of 500 μM PNDA, followed by the addition of GLY (15 mM) to inhibit CO_2 fixation. C_i (100 μM) was added after the addition of GLY as shown. Continuous actinic light was provided by a quartz-halogen lamp at 800 μmol m^{-2} s^{-1} PPFD. The rates of O_2 evolution are given as μmol mg^{-1} Chl h^{-1}. The light was turned off (at D) and intracellular C_i was allowed to leak into the reaction medium. The intracellular C_i pool was calculated as described in "Materials and Methods." [Chl] was 12.3 μg mL^{-1}.

developed with O_2 as the electron acceptor (Li and Canvin, 1997a).

The intracellular C_i accumulation with MV as the electron acceptor under various conditions is shown in Figure 7. The experiments were carried out in the presence of 1 mM MV and aerobic conditions, 1 mM MV and anaerobic conditions, and anaerobic conditions without MV. With MV in aerobic conditions only a small (20 mM) intracellular pool developed (Fig. 7A). When O_2 was excluded a much larger pool developed (54 mM; Fig. 7B), but the C_i pool was largest (66 mM) in the absence of both MV and O_2. The significance of these results is that intracellular C_i pools are formed in the presence of either MV and PNDA and that these pools stimulate electron transport through PSI.

Effect of C_i Concentration on O_2 Evolution from PNDA Photoreduction

The response of O_2 evolution with PNDA as the electron acceptor in air-grown cells of *Synechococcus* UTEX 625 in relation to the extracellular C_i concentration and intracellular C_i pool size is shown in Figure 8 under conditions of inhibited CO_2 fixation. It is apparent that in the absence of C_i, O_2 evolution was severely inhibited and no net O_2 evolution was observed (Fig. 8), even though most of the PNDA was present in its oxidized form (Fig. 6). The addition of C_i to cells stimulated the rate of O_2 evolution. Significant O_2 evolution occurred even upon addition of very low C_i concentrations. As C_i concentration was increased to 50 μM, O_2 evolution increased, but above this C_i concentration there was no significant increase in O_2 evolution. The maximum rate of O_2 evolution was 208 μmol

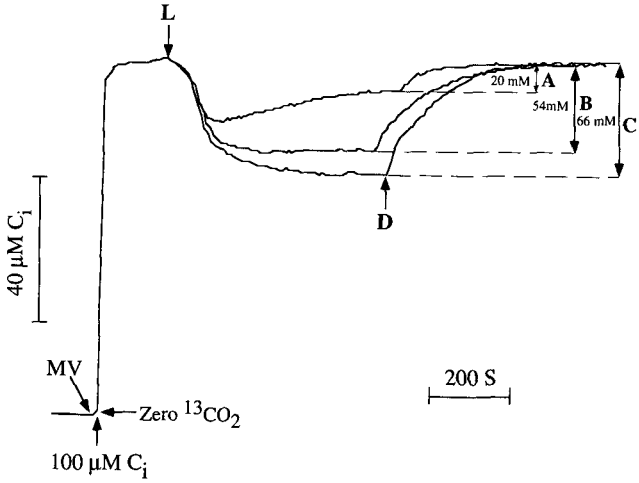

Figure 7. Typical patterns of changes in the extracellular [CO_2] monitored by MS with air-grown cells of *Synechococcus* UTEX 625 in the presence of 1 mM MV and 240 μM O_2 (A), MV and anaerobic conditions (B), and anaerobic conditions without MV (C). Cells were suspended at 30°C in BTP-HCl buffer, pH 8.0, illuminated with a light intensity of 300 μmol m^{-2} s^{-1} in the presence of 25 mM NaCl and 25 μg mL^{-1} CA, and allowed to reach the CO_2 compensation point. GLY (15 mM) was added to inhibit CO_2 fixation 4 min before the addition of MV. The lights were turned off and 100 μM C_i was added 10 s later. Cells were dark-adapted for 1 min. The light was turned on as indicated (L), and the cells were allowed to take up the extracellular $^{13}C_i$ for about 10 min. The light was then turned off (at D) and the intracellular C_i was allowed to leak back into the reaction medium. [Chl] was 5.4 μg mL^{-1}.

mg^{-1} Chl h^{-1}. The maximum size of the intracellular C_i pool in this experiment was 28 mM. From these data the extracellular and intracellular $K_{1/2}$ values for PNDA-dependent O_2 evolution were calculated using double-reciprocal plots of rates versus concentration. The extracellular $K_{1/2}$ for O_2 evolution was about 3.8 μM C_i (Fig. 8A) and values of 4.0 and 8.4 μM were obtained in two other experiments. The intracellular $K_{1/2}$ was 1.4 mM C_i (Fig. 8B), but a value of 0.5 mM was observed in another experiment. These are in agreement with the previous values reported for the stimulation of O_2 photoreduction (Li and Canvin, 1997b).

Effect of C_i on Reduction of PSII Electron Acceptors

Electron transport through PSII was measured using DMQ plus ferricyanide (500 μM, each) (Badger and Schreiber, 1993) and oxidized DAD (200 μM) plus 1 μM 2,5-dibromo-3-methyl-6-isopropyl *p*-benzoquinone (Demeter et al., 1995). The light intensity was 400 μmol m^{-2} s^{-1} for O_2 evolution experiments and 80 μmol m^{-2} s^{-1} for fluorescence measurements. When DMQ was added to CO_2-depleted cells inhibited with GLY, $F'_m - F/F'_m$ was 0.41. When DADox was added to such cells, $F'_m - F/F'_m$ was 0.32. No further quenching occurred when 100 μM C_i was added to the reaction mixture. O_2 evolution from CO_2-depleted cells inhibited with GLY was 292 μmol mg^{-1} Chl h^{-1} when DMQ was added and 186 μmol mg^{-1} Chl h^{-1} when DADox was added. No stimulation of O_2 evolution was observed when 100 μm C_i was added.

DISCUSSION

For air-grown cells of *Synechococcus* UTEX 625 it has been shown that O_2 can act as an efficient electron acceptor for electrons derived from the photooxidation of water under conditions in which CO_2 fixation is allowed or inhibited (Miller et al., 1988a, 1991; Canvin et al., 1990; Li and Canvin, 1997a, 1997b). The electrons required for this O_2 photoreduction are derived from PSII, but the actual electron donation to O_2 probably occurs at PSI (Canvin et al., 1990; Badger

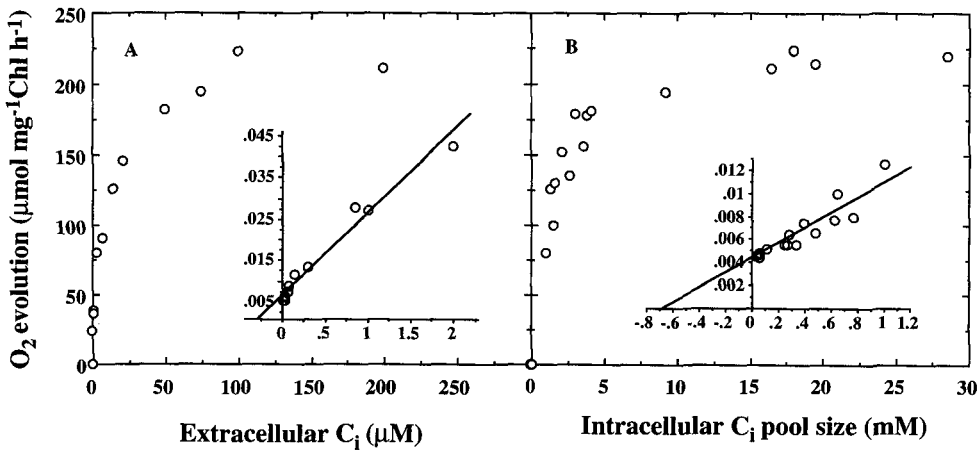

Figure 8. The magnitude of O_2 evolution with PNDA as an electron acceptor as a function of the extracellular C_i concentration (A) and the intracellular C_i pool (B) in cells of *Synechococcus* UTEX 625. Cells were suspended in 25 mM BTP-HCl buffer, pH 8.0, and were allowed to reach the CO_2 compensation point. PNDA was introduced and experiments were carried out as described in Figure 6. The reaction mixture contained 25 mM NaCl and 25 μg mL^{-1} CA. Actinic light was 800 μmol m^{-2} s^{-1}. The reaction was started by the addition of various amounts of [C_i]. The insets show the double-reciprocal plots of velocity versus extracellular C_i (A) or intracellular C_i pool concentration (B). [Chl] was 5.5 to 8.0 μg mL^{-1}. The data presented are representative of three (A) and two (B) replicates.

and Schreiber, 1993). The photoreduction of O_2 is a controlled process that is stimulated by the accumulation of an intracellular C_i pool. The addition of C_i to GLY-inhibited cells at the CO_2 compensation point caused significant O_2-dependent photochemical quenching and a calculated electron flow rate 60 to 70% of that previously calculated when both CO_2 and O_2 were possible electron acceptors.

The site at which intracellular C_i stimulates electron flow to O_2 remains unknown. Previously, it was assumed (Miller et al., 1988a, 1991; Canvin et al., 1990) that the C_i affected some site prior to Fd in the electron transport chain and that O_2 was reduced via Fd. The flow of electrons in the two photosystems of isolated thylakoids from higher plants has been studied extensively using artificial electron acceptors (Izawa, 1980). When artificial electron acceptors were applied to study electron flow in cyanobacteria, however, no effect of C_i could be determined on electron transfer in either PSII or PSI (Badger and Schreiber, 1993). The authors concluded that C_i was in some way directly stimulating the reduction of O_2. This mechanism of action of C_i, however, did not seem adequate when it was discovered that C_i also stimulated the reduction of NO_2^- (Mir et al., 1995a), because it would require a direct effect of C_i on the reduction of NO_2^-. Consequently, it was considered necessary to re-investigate the effect of C_i on the reduction of artificial electron acceptors in cyanobacteria, especially considering that very low concentrations of extracellular C_i were sufficient to substantially stimulate the reduction of O_2 (Li and Canvin, 1997b).

When using artificial electron acceptors in isolated thylakoids the presence of O_2 or CO_2 can be largely ignored. In intact cyanobacteria, however, this is not so because O_2 and CO_2 can act as electron acceptors and CO_2 (C_i) can stimulate electron flow. PNDA at 200 μM or greater totally prevented O_2 photoreduction at all light intensities (Fig. 2). This concentration of PNDA completely inhibited CO_2 fixation at low light intensity but resulted in only partial inhibition at higher light intensities (Fig. 2). Somewhat similar results have been observed with NO_2^- addition (Mir et al., 1995b); at 300 μmol m^{-2} s^{-1} PPFD, NO_2^- did not inhibit CO_2 fixation but reduced O_2 photoreduction by 60%. At 80 μmol m^{-2} s^{-1} light intensity NO_2^- inhibited both CO_2 fixation (17%) and O_2 photoreduction (28%). These results indicate that the flow of electrons to CO_2 fixation is less sensitive to inhibition by NO_2^- and PNDA than O_2 photoreduction. This may indicate a difference in some step of electron transport to CO_2 and to O_2. One should also note that at higher light intensities saturating C_i does not completely inhibit O_2 photoreduction (Fig. 2).

There is little doubt that PNDA was an efficient alternative electron acceptor to O_2 (Figs. 1 and 2). Electron transfer to PNDA was also greatly stimulated by the addition of C_i (Fig. 3). The rate of electron transfer to PNDA and the degree of stimulation of electron transfer by C_i, however, was sensitive to light intensity and to the order of addition of PNDA and the CO_2 fixation inhibitor GLY. When PNDA was added first, it did not totally prevent CO_2 fixation, and any CO_2 added with the PNDA would be quickly fixed in photosynthesis. In spite of all precautions it would seem that some C_i had been added with the PNDA solution. When GLY was subsequently added to inhibit CO_2 fixation, the addition of C_i then stimulated PNDA reduction by severalfold (Fig. 3). When GLY was added first, followed by PNDA, the initial rate of PNDA reduction was higher and stimulation by C_i was less (Fig. 3).

When cells of *Synechococcus* UTEX 625 are incubated in the presence of 25 mM NaCl and CA they can be quickly taken to a very low CO_2 compensation point. At this point the cells exhibit high levels of Chl *a* fluorescence and low rates of electron flow. When CO_2 fixation was inhibited in such cells, the addition of PNDA or MV resulted in substantial quenching (Figs. 4 and 5). The addition of C_i increased the quenching due to PNDA in both aerobic and anaerobic conditions (Fig. 4). In aerobic conditions MV caused substantial quenching and the addition of C_i had no effect (Fig. 5A). This is similar to the results of Badger and Schreiber (1993). In anaerobic conditions, however, quenching due to MV addition was observed only after C_i addition (Fig. 5B). The lack of quenching due to MV in anaerobic conditions in spinach chloroplasts was ascribed to the reduction of plastoquinone via cyclic electron transfer from the MV radicals (Asada et al., 1990). Such an explanation does not seem to be applicable to our results with cyanobacteria (Fig. 5), because it would not be compatible with the stimulation of electron flow through PSI that results from the addition of C_i (Fig. 5B). Presumably, before the addition of C_i cyclic electron flow through PSI would be inhibited; therefore cyclic flow from the MV radical would not be expected.

Although fluorescence quenching and net O_2 evolution from PNDA addition can be observed in the absence of C_i, both of these features are increased by the addition of C_i (Figs. 3 and 4). Quenching due to MV in anaerobic conditions only occurs upon the addition of C_i (Fig. 5B). The stimulation of electron flow to O_2 by C_i has previously been attributed to the formation of intracellular C_i pools rather than to the transport of C_i (Miller et al., 1990, 1991; Badger and Schreiber, 1993). Small but significant intracellular pools of C_i are formed in the presence of PNDA (Fig. 6) or MV (Fig. 7).

The degree of O_2 evolution and the magnitude of fluorescence quenching induced by the photoreduction of PNDA was dependent on the C_i concentration. The extracellular $K_{1/2}$ was 3.8 μM and the intracellular $K_{1/2}$ was 1.4 mM, and these values agree closely with the $K_{1/2}$ values of extracellular and intracellular C_i for O_2 photoreduction (Li and Canvin, 1997b).

In agreement with the observations of Badger and Schreiber (1993), we found that pronounced photochemical quenching and O_2 evolution were induced by the addition of the PSII acceptors DMQ and ferricyanide or DADox plus 2,5-dibromo-3-methyl-6-isopropyl *p*-benzoquinone in the absence of C_i.

The function of O_2 photoreduction by cyanobacteria remains unknown, although various authors have considered the possibility that it may be involved in the pseudocyclic synthesis of the ATP required for the active transport of C_i for CO_2 fixation (Miller et al., 1988a; Canvin et al., 1990; Sültemeyer et al., 1993). It is believed that PSI activity provides the energy for sustained transport (Ogawa and

Inoue, 1985; Li and Canvin, 1997a). We found that when electrons were allowed to drain into electron acceptors of PSI such as PNDA and MV significant C_i transport and accumulation by *Synechococcus* UTEX 625 occurred (Figs. 6 and 7). These results suggest that under these conditions the ATP produced as a result of linear electron flow was sufficient to drive C_i transport.

We recognize that our results contradict the observations of Badger and Schreiber (1993), who were not able to obtain any effect of added C_i on electron transfer to PNDA or MV, and it is not possible to fully reconcile the findings. Badger and Schreiber (1993) used a different strain of *Synechococcus* and red actinic light at 250 μmol m^{-2} s^{-1}, and they added GLY before adding the artificial electron acceptors. We have found that the reduction of PNDA can be completely inhibited in the absence of C_i when PNDA is added to the cells prior to GLY in saturating light conditions (greater than 500 μmol m^{-2} s^{-1}). The addition of C_i at that time results in a large stimulation of PNDA reduction. Under aerobic conditions our results with MV are the same as those of Badger and Schreiber (1993) but under anaerobic conditions MV causes only quenching upon the addition of C_i.

Taken together with the effects of C_i on O_2 photoreduction (Miller et al., 1988a; Li and Canvin, 1997a, 1997b) and NO_2^- reduction (Mir et al., 1995a, 1995b), the effects of C_i on PNDA and MV reduction suggest that C_i acts to release linear electron transport at some site in PSI or in the intersystem electron transport chain. This suggestion is further supported by the fact that PNDA reduction and O_2 photoreduction respond similarly to C_i concentrations.

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