

# Inorganic Carbon Accumulation Stimulates Linear Electron Flow to Artificial Electron Acceptors of Photosystem I in Air-Grown Cells of the Cyanobacterium *Synechococcus* UTEX 625<sup>1</sup>

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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  $\mu M$  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

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.

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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  $\mu mol m^{-2} s^{-1}$ . 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  $\mu g mL^{-1}$ , the  $C_i$  was about 50  $\mu M$ , 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 10,000g in a microfuge at room temperature. Cells were resuspended at 5 to 8  $\mu g Chl mL^{-1}$  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  $\mu mol m^{-2} s^{-1}$  light in the presence of 25 mM NaCl and 25  $\mu g mL^{-1}$  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  $\mu mol m^{-2} s^{-1}$ . The pulse measuring beam (100 kHz) was 1  $\mu mol m^{-2} s^{-1}$ . 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  $\mu mol m^{-2} s^{-1}$ . 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  $\mu g mL^{-1}$  GOD, and 50  $\mu g mL^{-1}$  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  $\mu mol mg^{-1} Chl h^{-1}$ ). 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,  $F_{mv}^*$  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  $[C_e]$  in the light and the  $[C_i]$  that occurred upon darkening the cells. The internal  $[C_i]$  was calculated using an intracellular volume of  $75 \mu\text{L mg}^{-1}$  Chl (Miller et al., 1988c).

### Measurement of Photoreduction of PNDA

PNDA photoreduction was determined spectrophotometrically using an extinction coefficient of  $3.4 \times 10^4$  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.  $K_2^{13}\text{CO}_3$  (99 atom%  $^{13}\text{C}$ ) was obtained from MSD Isotopes (Montreal, Quebec, Canada). The  $\text{KH}^{13}\text{CO}_3$  was dissolved in BTP-HCl, pH 8.0, as a concentrated solution for addition to the reaction medium (Miller et al., 1988b).  $^{18}\text{O}_2$  (97.4 atom%  $^{18}\text{O}$ ) was supplied by Cambridge Isotope Laboratories (Andover, MA).

## RESULTS

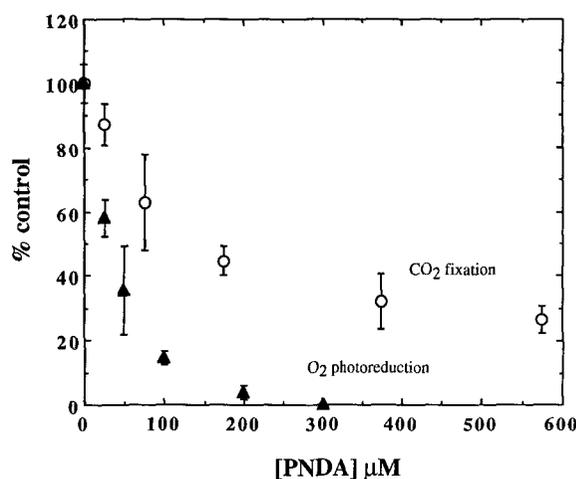
### Effect of PNDA on $\text{CO}_2$ Fixation and $\text{O}_2$ Photoreduction

Previous work on  $\text{O}_2$  photoreduction,  $\text{NO}_2^-$  reduction, and  $\text{CO}_2$  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  $\text{O}_2$  photoreduction and  $\text{CO}_2$  fixation.

$\text{O}_2$  photoreduction in GLY-inhibited cells was very sensitive to PNDA addition and was totally inhibited by  $200 \mu\text{M}$  PNDA (Fig. 1).  $\text{CO}_2$  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  $\text{O}_2$  photoreduction by  $200 \mu\text{M}$  PNDA did not change over the range of light intensity investigated whether or not  $\text{CO}_2$  fixation was inhibited (Fig. 2). However, the inhibition of  $\text{CO}_2$  fixation by  $200 \mu\text{M}$  PNDA did change with light intensity. At low light intensity ( $80 \mu\text{mol m}^{-2} \text{s}^{-1}$ ),  $\text{CO}_2$  fixation was inhibited 100%, but as the light intensity was increased the inhibition decreased to about 50% (Fig. 2, A and C).

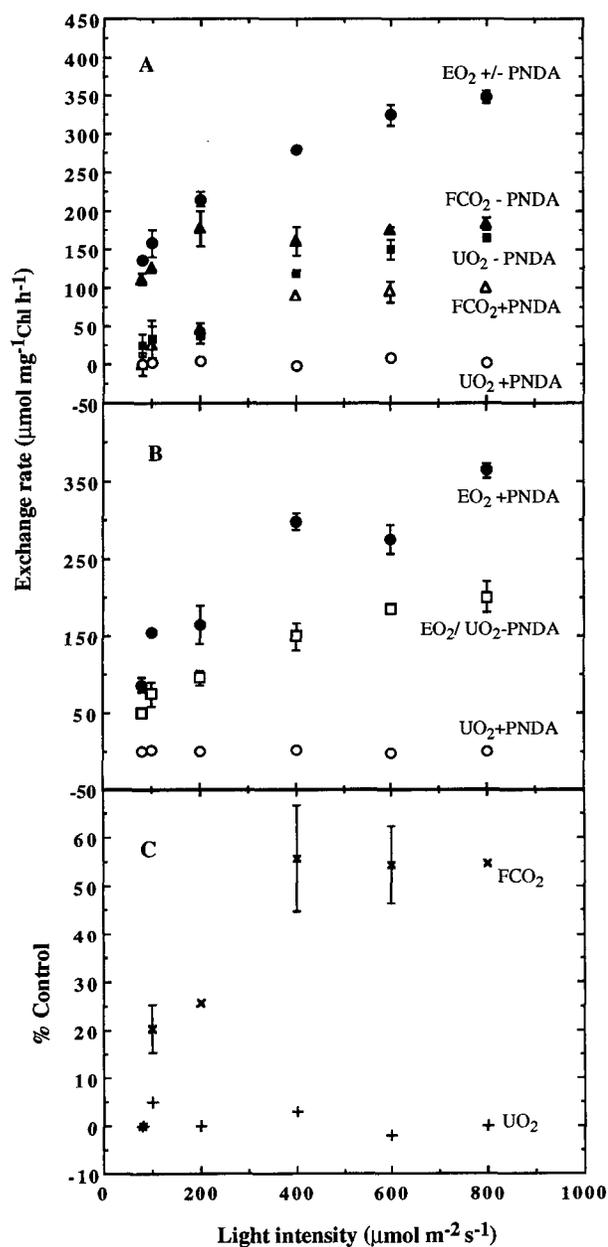
When  $\text{CO}_2$  fixation was not inhibited (Fig. 2A), the addition of PNDA as another electron acceptor did not increase the rate of  $\text{O}_2$  evolution, even at the highest light intensity used. When  $\text{CO}_2$  fixation was inhibited, however,  $\text{O}_2$  evolution with only  $\text{O}_2$  as the electron acceptor (Fig. 2B, -PNDA) was only 50% of that in noninhibited cells (compare  $\text{EO}_2 - \text{PNDA}$ , Fig. 2A, with  $\text{EO}_2 - \text{PNDA}$ , Fig. 2B). The added electron-accepting capacity of PNDA restored  $\text{O}_2$  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  $\text{CO}_2$  fixation and  $\text{O}_2$  photoreduction at a light intensity of  $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Rates of  $\text{CO}_2$  fixation and  $\text{O}_2$  photoreduction were measured as described in "Materials and Methods." The rate of  $\text{CO}_2$  fixation in the absence of PNDA was  $254 \mu\text{mol mg}^{-1} \text{Chl h}^{-1}$  with  $200 \mu\text{M } C_i$ , and the inhibitory effect of PNDA on  $\text{CO}_2$  fixation (O) was measured after successive additions of PNDA to yield increasingly higher concentrations. The control rate of  $\text{O}_2$  photoreduction was  $168 \mu\text{mol mg}^{-1} \text{Chl h}^{-1}$  ( $\blacktriangle$ ) and was measured after the addition of  $100 \mu\text{M } C_i$  in the light in the presence of  $15 \text{ mM GLY}$ . PNDA in increasing concentrations was then added to inhibit  $\text{O}_2$  photoreduction. The  $[\text{Chl}]$  was  $5.5 \mu\text{g mL}^{-1}$ . The data shown are means  $\pm$  SD ( $n = 3$ ).

in the light can deplete the  $[C_e]$  in the medium to 0 levels. If  $\text{CO}_2$  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  $1.0 \mu\text{M } C_i$  in the extracellular medium will greatly stimulate  $\text{O}_2$  photoreduction (Li and Calvin, 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  $\text{CO}_2$  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  $\text{O}_2$  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 ( $80 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and GLY was added before PNDA.

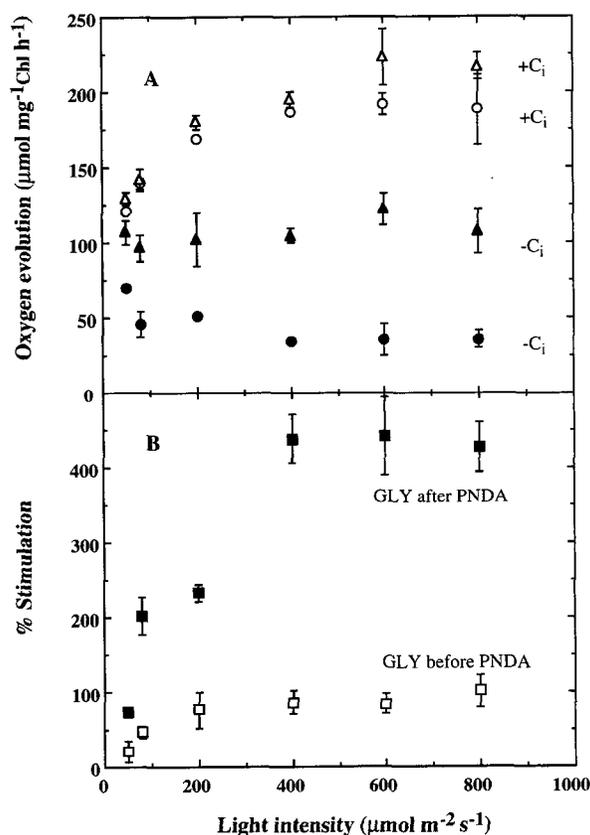


**Figure 2.** The relationship between light intensity and the effect of PANDA ( $200 \mu\text{M}$ ) addition on gas exchange of noninhibited cells (A), on gas exchange in which  $\text{CO}_2$  fixation was inhibited (B), and on the percentage of  $\text{CO}_2$  fixation or  $\text{O}_2$  photoreduction (from A) remaining after PANDA addition (C). In A, gross  $\text{O}_2$  evolution ( $\bullet$ ,  $\text{EO}_2$ ),  $\text{CO}_2$  fixation ( $\Delta$ ,  $\blacktriangle$ ,  $\text{FCO}_2$ ), and  $\text{O}_2$  photoreduction ( $\circ$ ,  $\blacksquare$ ,  $\text{UO}_2$ ) were measured before ( $-$ PANDA) and after ( $+$ PANDA) PANDA addition. In B,  $\text{O}_2$  photoreduction ( $\circ$ ,  $\square$ ,  $\text{UO}_2$ ) and evolution ( $\bullet$ ,  $\square$ ,  $\text{EO}_2$ ) in the presence of  $15 \text{ mM}$  GLY (added before  $\text{C}_i$ ) were measured before ( $-$ PANDA) and after ( $+$ PANDA) addition. In C, the remaining percentage of the rates of  $\text{CO}_2$  fixation ( $\times$ ,  $\text{FCO}_2$ ) and  $\text{O}_2$  photoreduction ( $+$ ,  $\text{UO}_2$ ) in the presence of PANDA are shown. The  $\text{O}_2$  concentration was  $240 \mu\text{M}$ . The  $\text{C}_i$  was added in the light at  $200 \mu\text{M}$  (A) or  $100 \mu\text{M}$  (B). The rates of  $\text{CO}_2$  fixation and  $\text{O}_2$  exchange were measured by MS and calculated as described in "Materials and Methods." The  $[\text{Chl}]$  was  $5.3 \mu\text{g mL}^{-1}$ . The data shown are means  $\pm$  SD ( $n = 3$ ).

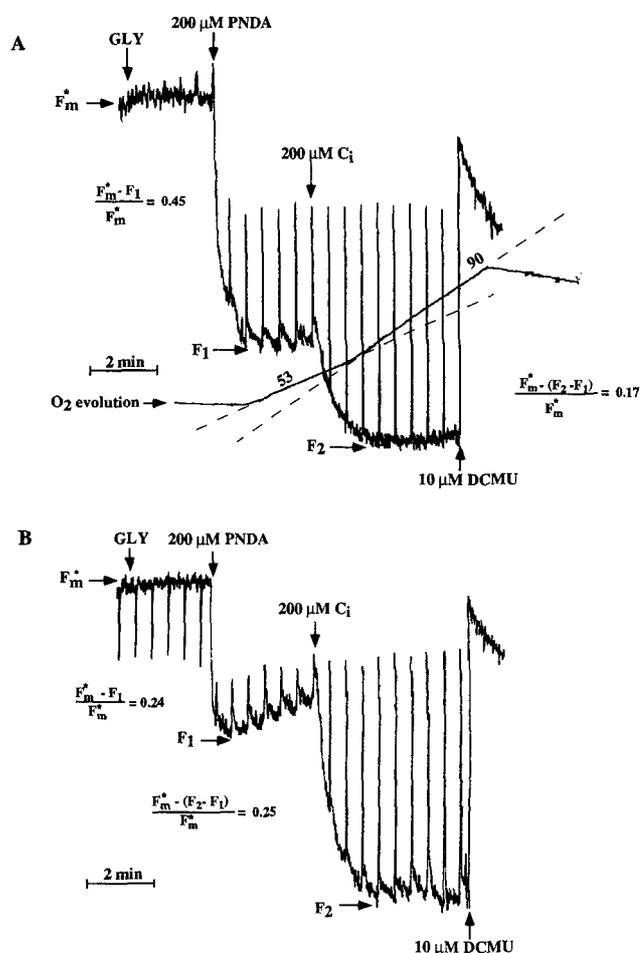
### $\text{C}_i$ Effect on PANDA and MV-Induced Quenching

PANDA and MV accept electrons from PSI (Elstner and Zeller, 1978; Fujii et al., 1990), and the abilities of PANDA and MV to act as electron acceptors in the absence of  $\text{C}_i$  were examined. The experiments were carried out in the presence (Figs. 4A and 5A) or absence (Figs. 4B and 5B) of  $\text{O}_2$  under conditions in which  $\text{CO}_2$  fixation was inhibited with GLY (Miller and Canvin, 1989)

Figure 4 shows the effect of  $\text{C}_i$  on PANDA-induced quenching of Chl *a* fluorescence under aerobic and anaerobic conditions. The rate of  $\text{O}_2$  evolution due to PANDA photoreduction was also measured (Fig. 4A). In the absence of  $\text{C}_i$ , at the  $\text{CO}_2$  compensation point the addition of  $200 \mu\text{M}$  PANDA initiated quenching of Chl *a* fluorescence and  $\text{O}_2$  evolution simultaneously (Fig. 4A). Quenching also occurred in the absence of  $\text{C}_i$  under anaerobic conditions (Fig. 4B). The addition of  $\text{C}_i$  resulted in a 40% increase in



**Figure 3.** The effect of light intensity on  $\text{O}_2$  evolution (A) and the percentage stimulation of  $\text{O}_2$  evolution by the addition of  $\text{C}_i$  (B) with PANDA as the electron acceptor. Cells were incubated in the light to reach the  $\text{CO}_2$  compensation point, and  $\text{O}_2$  evolution due to PANDA reduction was measured using the  $\text{O}_2$  electrode. The reactions were started by the addition of  $200 \mu\text{M}$  PANDA, followed by  $200 \mu\text{M}$   $\text{C}_i$ . The initial  $\text{O}_2$  was  $80 \mu\text{M}$ .  $\text{O}_2$  evolution was calculated as described in "Materials and Methods."  $\text{CO}_2$  fixation was inhibited with  $15 \text{ mM}$  GLY either 2 min before ( $\Delta$ ,  $\blacktriangle$ ) or 5 min after ( $\circ$ ,  $\bullet$ ) the addition of PANDA (A). The stimulation of PANDA reduction by  $\text{C}_i$  (B), in which GLY was added before ( $\square$ ) or after ( $\blacksquare$ ) PANDA is shown. The data shown are means  $\pm$  SD for three separate measurements with the same cells ( $5.5 \mu\text{g Chl mL}^{-1}$ ).



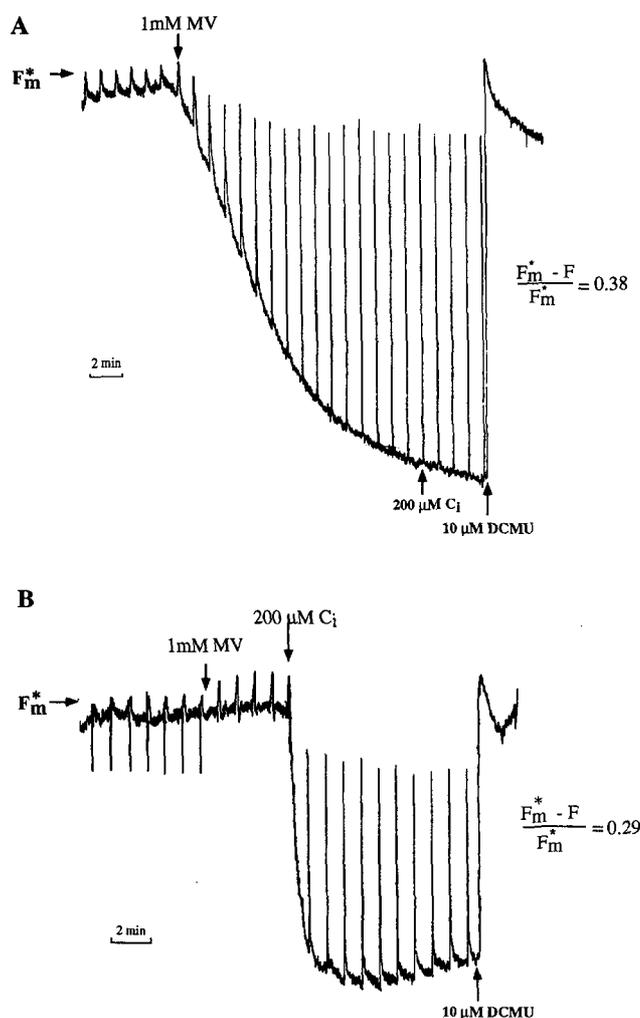
**Figure 4.** The effect of C<sub>i</sub> on PNDA-dependent quenching of Chl *a* fluorescence and O<sub>2</sub> evolution under aerobic (A) and anaerobic (B) conditions in cells of *Synechococcus* UTEX 625. The cells were preincubated in the light to remove C<sub>i</sub> from the medium in the presence of 25 mM NaCl and 25 μg mL<sup>-1</sup> 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<sub>i</sub> as shown. Actinic light was 80 μmol m<sup>-2</sup> s<sup>-1</sup>. The initial O<sub>2</sub> concentration was 150 μM and O<sub>2</sub> evolution was recorded using an O<sub>2</sub> electrode (A). Rates of O<sub>2</sub> evolution are given as μmol mg<sup>-1</sup> Chl h<sup>-1</sup> (the numbers next to the trace). When required, O<sub>2</sub> was removed from the reaction mixture (B) by the addition of GOD. [Chl] was 6.6 μg mL<sup>-1</sup>. 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<sub>i</sub> was transiently relieved when cells were illuminated with a saturating flash of light, indicating that the quenching caused by the addition of C<sub>i</sub> was largely photochemical quenching (Fig. 4). When O<sub>2</sub> evolution was measured (Fig. 4A), the addition of C<sub>i</sub> stimulated O<sub>2</sub> 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<sub>m</sub><sup>\*</sup>), 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<sub>2</sub> (Fig. 5A). Quenching was maximal even in the absence of C<sub>i</sub> (Fig. 5A), and the calculated electron flow rate was equivalent to the rate when both CO<sub>2</sub> and O<sub>2</sub> were possible electron acceptors (Li and Carvin, 1997a). Under aerobic conditions the photoreduction of MV, as determined from O<sub>2</sub> uptake with MS, proceeded at a high and constant rate of 200 μmol mg<sup>-1</sup> Chl h<sup>-1</sup> even in the absence of C<sub>i</sub> (data not shown). The addition of C<sub>i</sub> 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<sub>i</sub> 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<sub>2</sub>. Cell suspensions (5.1 μg Chl mL<sup>-1</sup>) were incubated as described in "Materials and Methods" and allowed to reach the CO<sub>2</sub> compensation point. Photosynthesis was inhibited by the addition of 15 mM GLY. O<sub>2</sub> was removed from the medium (B) by the addition of the GOD. The initial O<sub>2</sub> concentration was 150 μM. Actinic light was 80 μmol m<sup>-2</sup> s<sup>-1</sup>. The reaction was started by the addition of 1 mM MV. At the times indicated, 200 μM C<sub>i</sub> 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).

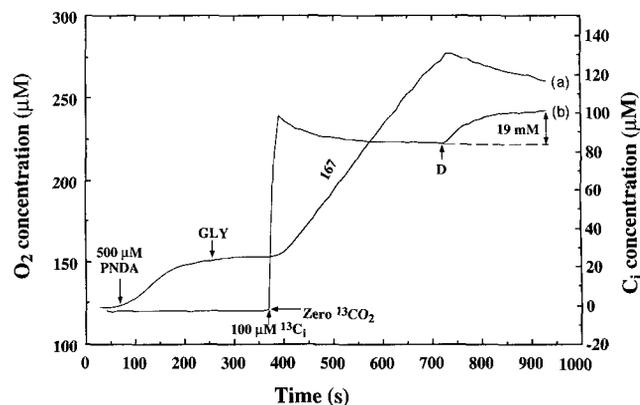
#### Suppression of Photoreduction of PNDA in the Absence of $C_i$ and $C_i$ Accumulation during Photoreduction of PNDA and MV

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  $500 \mu\text{mol m}^{-2} \text{s}^{-1}$  (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 ( $167 \mu\text{mol mg}^{-1} \text{Chl h}^{-1}$ ; Fig. 6).

The rate of photoreduction of PNDA was also determined from the decrease in  $A_{440}$ . A higher rate of photoreduction of PNDA ( $208 \mu\text{mol mg}^{-1} \text{Chl h}^{-1}$ ) was observed in the presence of  $C_i$  compared with the rate ( $5 \mu\text{mol mg}^{-1} \text{Chl h}^{-1}$ ) 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



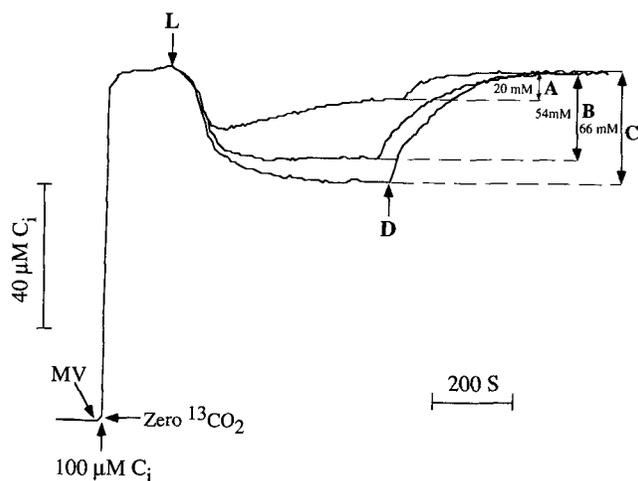
**Figure 6.**  $C_i$  effect on PNDA-dependent  $O_2$  evolution (a) and  $C_i$  accumulation (b) monitored by MS with air-grown cells of *Synechococcus* UTEX 625. The cells were preincubated in the light to remove  $C_i$  from the medium in the presence of 25 mM NaCl and  $25 \mu\text{g mL}^{-1}$  CA. The reaction was started by the addition of  $500 \mu\text{M}$  PNDA, followed by the addition of GLY (15 mM) to inhibit  $CO_2$  fixation.  $C_i$  ( $100 \mu\text{M}$ ) was added after the addition of GLY as shown. Continuous actinic light was provided by a quartz-halogen lamp at  $800 \mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD. The rates of  $O_2$  evolution are given as  $\mu\text{mol mg}^{-1} \text{Chl h}^{-1}$ . 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  $12.3 \mu\text{g mL}^{-1}$ .

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  $50 \mu\text{M}$ ,  $O_2$  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  $208 \mu\text{mol}$



**Figure 7.** Typical patterns of changes in the extracellular  $[^{13}\text{C}_i]$  monitored by MS with air-grown cells of *Synechococcus* UTEX 625 in the presence of 1 mM MV and 240  $\mu\text{M}$   $\text{O}_2$  (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  $\mu\text{mol m}^{-2} \text{s}^{-1}$  in the presence of 25 mM NaCl and 25  $\mu\text{g mL}^{-1}$  CA, and allowed to reach the  $\text{CO}_2$  compensation point. GLY (15 mM) was added to inhibit  $\text{CO}_2$  fixation 4 min before the addition of MV. The lights were turned off and 100  $\mu\text{M}$   $\text{C}_i$  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  $^{13}\text{C}_i$  for about 10 min. The light was then turned off (at D) and the intracellular  $\text{C}_i$  was allowed to leak back into the reaction medium.  $[\text{Chl}]$  was 5.4  $\mu\text{g mL}^{-1}$ .

$\text{mg}^{-1} \text{Chl h}^{-1}$ . The maximum size of the intracellular  $\text{C}_i$  pool in this experiment was 28 mM. From these data the extracellular and intracellular  $K_{1/2}$  values for PNDA-dependent  $\text{O}_2$  evolution were calculated using double-reciprocal plots of rates versus concentration. The extracellular  $K_{1/2}$  for  $\text{O}_2$

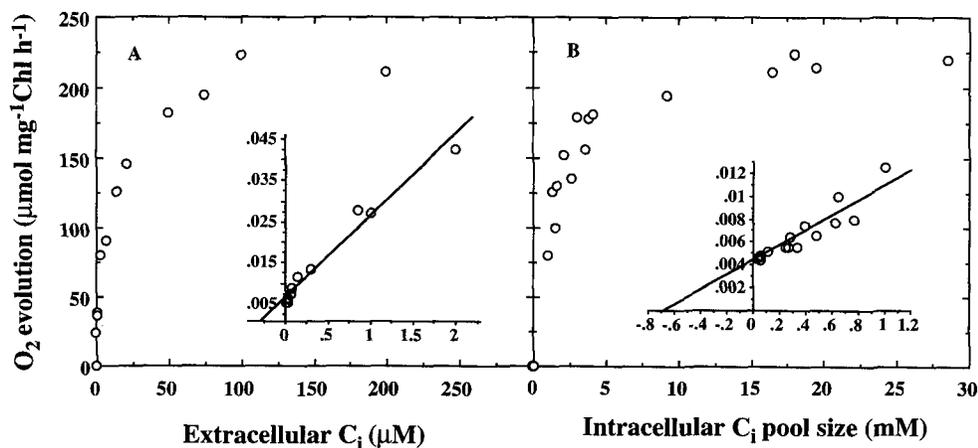
evolution was about 3.8  $\mu\text{M}$   $\text{C}_i$  (Fig. 8A) and values of 4.0 and 8.4  $\mu\text{M}$  were obtained in two other experiments. The intracellular  $K_{1/2}$  was 1.4 mM  $\text{C}_i$  (Fig. 8B), but a value of 0.5 mM was observed in another experiment. These are in agreement with the previous values reported for the stimulation of  $\text{O}_2$  photoreduction (Li and Calvin, 1997b).

### Effect of $\text{C}_i$ on Reduction of PSII Electron Acceptors

Electron transport through PSII was measured using DMQ plus ferricyanide (500  $\mu\text{M}$ , each) (Badger and Schreiber, 1993) and oxidized DAD (200  $\mu\text{M}$ ) plus 1  $\mu\text{M}$  2,5-dibromo-3-methyl-6-isopropyl *p*-benzoquinone (Demeter et al., 1995). The light intensity was 400  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for  $\text{O}_2$  evolution experiments and 80  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for fluorescence measurements. When DMQ was added to  $\text{CO}_2$ -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  $\mu\text{M}$   $\text{C}_i$  was added to the reaction mixture.  $\text{O}_2$  evolution from  $\text{CO}_2$ -depleted cells inhibited with GLY was 292  $\mu\text{mol mg}^{-1} \text{Chl h}^{-1}$  when DMQ was added and 186  $\mu\text{mol mg}^{-1} \text{Chl h}^{-1}$  when DADox was added. No stimulation of  $\text{O}_2$  evolution was observed when 100  $\mu\text{M}$   $\text{C}_i$  was added.

### DISCUSSION

For air-grown cells of *Synechococcus* UTEX 625 it has been shown that  $\text{O}_2$  can act as an efficient electron acceptor for electrons derived from the photooxidation of water under conditions in which  $\text{CO}_2$  fixation is allowed or inhibited (Miller et al., 1988a, 1991; Calvin et al., 1990; Li and Calvin, 1997a, 1997b). The electrons required for this  $\text{O}_2$  photoreduction are derived from PSII, but the actual electron donation to  $\text{O}_2$  probably occurs at PSI (Calvin et al., 1990; Badger



**Figure 8.** The magnitude of  $\text{O}_2$  evolution with PNDA as an electron acceptor as a function of the extracellular  $\text{C}_i$  concentration (A) and the intracellular  $\text{C}_i$  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  $\text{CO}_2$  compensation point. PNDA was introduced and experiments were carried out as described in Figure 6. The reaction mixture contained 25 mM NaCl and 25  $\mu\text{g mL}^{-1}$  CA. Actinic light was 800  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . The reaction was started by the addition of various amounts of  $[\text{C}_i]$ . The insets show the double-reciprocal plots of velocity versus extracellular  $\text{C}_i$  (A) or intracellular  $\text{C}_i$  pool concentration (B).  $[\text{Chl}]$  was 5.5 to 8.0  $\mu\text{g mL}^{-1}$ . 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  $\mu M$  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  $\mu mol\ m^{-2}\ s^{-1}$  PPFD,  $NO_2^-$  did not inhibit  $CO_2$  fixation but reduced  $O_2$  photoreduction by 60%. At 80  $\mu mol\ m^{-2}\ s^{-1}$  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  $\mu M$  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  $250 \mu\text{mol m}^{-2} \text{s}^{-1}$ , 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  $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). 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  $\text{NO}_2^-$  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 inter-system 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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