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Rapid communication

Bicarbonate effect on electron flow in a cyanobacterium *Synechocystis* PCC 6803

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Abstract. In this communication, evidence is presented from the kinetics of Q_A^- decay (where Q_A is the first plastoquinone electron acceptor of photosystem II) and oxygen evolution for the requirement of bicarbonate in the electron transport in a cyanobacterium *Synechocystis* (Pasteur Culture Collection 6803). A large slowing down of Q_A^- oxidation, measured from the variable chlorophyll *a* fluorescence after saturating actinic flashes, was observed in the thylakoids of *Synechocystis* 6803 depleted of bicarbonate in the presence of 25 mM formate. Qualitatively similar results were obtained with DCMU-treated thylakoids. This shows that bicarbonate depletion inhibits electron transport on the acceptor side of photosystem II between Q_A and the plastoquinone (PQ) pool in cyanobacteria. Addition of 2.5 mM HCO_3^- fully reversed the inhibition of electron flow caused by bicarbonate depletion. Two exponential phases of Q_A^- decay, a fast one and a slow one, were observed with halftimes of approx. 400 μs (fast) and 26 ms (slow) at pH 6.5. At pH 7.5, these phases were approx. 330 μs (fast) and 21 ms (slow), respectively. The amplitude, but not the halftime, of the fast component decreased by about 70% (pH 6.5) or 50% (pH 7.5); this was accompanied by a concomittant increase in the slow phase. Twenty mM bicarbonate stimulated, by a factor of 4, the Hill reaction in bicarbonate-depleted *Synechocystis* cells. This effect is independent of CO_2 fixation as it was observed even in the presence of an inhibitor DBMIB.

Abbreviations: Chl – Chlorophyll; DBMIB – 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMQ – 2,5-dimethyl-p-benzoquinone; S_{13} – 5-Cl, 3-*t*-butyl, 2′-Cl, 4′-NO_2-salicylanilide; PCC – Pasteur culture collection

Introduction

The Hill reaction is significantly and reversibly inhibited in chloroplasts of higher plants depleted of bicarbonate (HCO_3^-) in the presence of formate (see reviews by Vermaas and Govindjee 1981, Blubaugh and Govindjee 1988a). A recent hypothesis (see Blubaugh and Govindjee 1988a) is that there are two major sites of 'bicarbonate effect' on the electron acceptor side

of photosystem II (PS II): (1) as a ligand to Fe^{2+} in Q_A–Fe–Q_B complex that keeps the D1 and D2 proteins in their proper functional conformation (see supportive data and arugments in Vermaas and Rutherford 1984, Michel and Deisenhofer 1988); (2) as a participant in the protonation of Q_B^-. Arginine has been proposed to be responsible for binding these HCO_3^- (Blubaugh and Govindjee 1988a). A knowledge of the specific binding sites of HCO_3^- in PS II will not only help in understanding of the role of bicarbonate, but also in clarifying the controversy regarding the site of bicarbonate action (electron acceptor versus electron donor side of PS II, see Stemler 1982). This can be attempted through the molecular genetic approach. In higher plants, however, no transformation system is available that could enable native genes to be removed and replaced with modified genes. On the other hand, in transformable photosynthetic bacteria, no 'bicarbonate effect' has been observed (R Shopes, D Blubaugh, CA Wraight and Govindjee 1987, unpublished). Therefore, we considered it important to look at the 'bicarbonate effect' in cyanobacteria, since they evolutionarily link the gap between photosynthetic bacteria and higher plants. They have the relative simplicity of being prokaryotic cells, but are fundamentally similar to oxygenic photosynthetic system found in the chloroplasts (Stanier and Cohen-Bazire 1977, Curtis and Haselkorn 1984, Williams and Chisholm 1987). The knowledge obtained from cyanobacteria will certainly shed light on the understanding of the photosynthetic mechanism in higher plants. We chose *Synechocystis* PCC 6803 for our study because molecular-genetic techniques have been developed for pinpointing the structure/function relationships in D1 and D2 proteins in this organism (Vermaas et al. 1988). Our ultimate goal is to use these techniques as a tool to test the hypothesis of bicarbonate binding sites and investigate the role of bicarbonate in photosystem II. Another advantage is that the unicellular cyanobacterium *Synechocystis* 6803 can be an easier intact system to work with to answer questions related to the 'bicarbonate effect' in vivo.

Work on the 'bicarbonate effect' in cyanobacteria is scarce. The only study, known to us, is by van Rensen and Vermaas (1981) who showed no bicarbonate dependence in *Anacystis nidulans*. However, in this communication, we report a remarkable reversible inhibition of Q_A^- decay (where Q_A is the first plastoquinone electron acceptor of PS II) after saturating actinic flashes in the thylakoids of *Synechocystis* 6803 depleted of bicarbonate in the presence of formate. Furthermore, greater than 4 fold stimulation of oxygen evolution was observed when bicarbonate was added to the bicarbonate-depleted intact cells of *Synechocystis* 6803. The inhibition of Q_A^- decay will be discussed in terms of kinetic components and pH dependence.

Materials and methods

Synechocystis PCC 6803 was obtained from Dr H.B. Parkrasi. It was grown in BG-11 medium (Rippka et al. 1979) at a temperature of 28 °C and under continuous illumination of 70 μmol photons $m^{-2} s^{-1}$. Light was provided by warm white fluorescent lamps (Westinghouse Electric Corporation) (65 μmol photons $m^{-2} s^{-1}$) and incandescent lamps (General Electric) (5 μmol photons $m^{-2} s^{-1}$). The liquid culture was constantly bubbled with air pre-humidified by bubbling through a 1.0% $CuSO_4$ solution; the latter prevented the growth of contaminating organisms in the flask water. Cells in logarithmic phase were used throughout our studies.

Thylakoid isolation was essentially as described by Vermaas et al. (1986). Cells were first suspended in a medium containing 20 mM HEPES (pH 7.5), 0.3 M sucrose, 2 mM $MgCl_2$, 0.1% BSA and 1 mM EDTA before they were broken. Thylakoids were suspended in a medium containing 20 mM HEPES (pH 7.5), 0.3 M sucrose, and 2 mM $MgCl_2$.

Bicarbonate-depleted and bicarbonate-recovered samples were obtained as described by Eaton-Rye and Govindjee (1988). Depletion medium contained 0.3 M sorbitol, 25 mM $NaHCO_2$, 10 mM NaCl, 5 mM $MgCl_2$ and 10 mM NaH_2PO_4 (pH 5.8). The bicarbonate depleted samples were incubated for 4 hours in the depleted medium (pH 5.8) over which N_2 gas was passed. The reaction medium contained 0.1 M sorbitol, 20 mM $NaHCO_2$, 10 mM NaCl, 5 mM $MgCl_2$, 0.1 μM gramicidin D and 20 mM NaH_2PO_4 (pH 6.5) or 20 mM HEPES (pH 7.5).

The kinetics of decay of variable chlorophyll *a* fluorescence were measured at 685 nm (10 nm bandwidth) by a weak measuring flash. The measuring flash was fired at variable times after each actinic flash. The actinic flash (FX-124, EG and G) and the measuring flash (Stroboslave 1593A, General Radio) were filtered with Corning blue (CS4-96) glass filters; both had a 2.5 μs duration at half-maximal peak. For further details see Eaton-Rye (1987) and Eaton-Rye and Govindjee (1988). Chlorophyll *a* concentration was 10 μg/ml.

Oxygen evolution was determined polarographically using a Yellow Spring Instrument Clark-type electrode in a saturating yellow light (for details see Blubaugh 1987). A combination of two electron acceptors of DMQ and $K_3Fe(CN)_6$ was used in our study. Here, DMQ acts as the electron acceptor and ferricyanide keeps the DMQ in the oxidized state. DBMIB was used to block electron flow beyond the plastoquinone pool, but before photosystem I (Trebst 1980). Other details are given under Results and Discussion.

Results and discussion

Figure 1 shows Q_A^- decay after 3 actinic flashes at pH 6.5 (A) and pH 7.5 (B) in *Synechocystis* 6803 thylakoids. Q_A^- concentration was estimated from variable fluorescence according to Joliot and Joliot (1964), using the formula given by Mathis and Paillotin (1981). The connection parameter (p) was assumed to be 0.5. $[Q_A^-]$ is arbitrarily defined as 1 when fluorescence reaches the maximum. At both the pH values, the HCO_3^- depletion significantly slowed down the Q_A^- decay. By adding 2.5 mM HCO_3^- to the depleted samples, the inhibition of Q_A^- oxidation was relieved and the decay curve was fully restored to that of the control thylakoids. Govindjee et al. (1976) had found a very large slowing down of Q_A^- decay after three or more flashes in HCO_3^- depleted higher plant chloroplasts. Robinson et al. (1984) demonstrated that the Q_A^- decays after three or more flashes were 36-fold slower in the bicarbonate-depleted chloroplasts. The results obtained after 1 and 2 flashes in bicarbonate-depleted thylakoids of the cyanobacterium showed similar inhibition of Q_A^- decay (data not shown).

After an odd number of flashes, the following equilibrium exists: $Q_A^- Q_B \rightleftharpoons Q_A Q_B^-$, but a biphasic decay is observed. This biphasic decay of Q_A^- in chloroplasts has been discussed by Robinson and Crofts (1983). After a flash, the fast phase is suggested to be due to the electron transport in centers that have Q_B bound before the actinic flash, while the slow phase is suggested to be a second order process involving the binding of Q_B from the PQ pool. Both the fast and the slow phases were observed in Q_A^- decay in the thylakoids of the cyanobacterium. The amplitudes and halftimes from four experiments calculated from Q_A^- decay data, up to 1 s, are shown in Table 1. At pH 6.5, halftimes were 403 $\pm$ 5 μs (fast) and 26 $\pm$ 1 ms (slow). At pH 7.5, somewhat shorter halftimes (331 $\pm$ 3 μs (fast) and 21 $\pm$ 1 ms (slow)] were observed. Bicarbonate depletion did not lead to significant changes in the halftimes of Q_A^- decay suggesting that these may be from centers that still had bound HCO_3^- (see Blubaugh and Govindjee 1988b). At pH 6.5, HCO_3^- depletion led to a decrease in the amplitude of the fast component from 42% to 14%. However, the amplitude of the slow component increased from 58% to 86%. At pH 7.5, HCO_3^- depletion led to a decrease in the amplitude of the fast copmponent from 56% to 26%. However, the slow component increased from 45% to 74%. After 2.5 mM HCO_3^- was added, the amplitudes were restored to those of the controls (Table 1) proving complete reversibility.

Five μM DCMU almost eliminated the decay of Q_A^- in thylakoids from *Synechocystis* (Fig. 2). This inhibitor is known to block the reoxidation of Q_A^- by displacing Q_B from its binding site (Velthuys 1981). Thus, by anology,

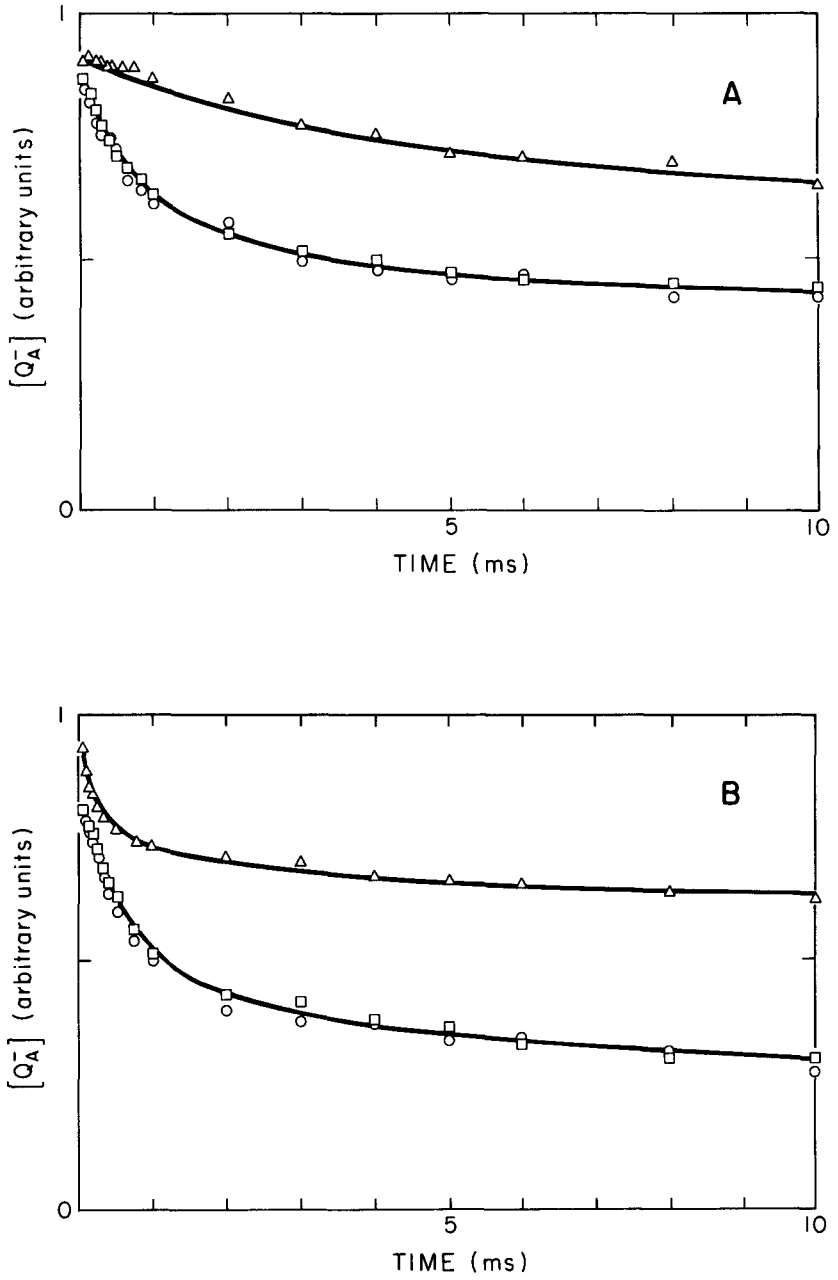

Fig. 1. Decay of Q_A^- after 3 flashes in thylakoids of *Synechocystis* 6803 at pH 6.5 (A) and pH 7.5 (B). The flash frequency was 1 Hz. (□) control; (△) bicarbonate-depleted; (○) restored by adding 2.5 mM HCO_3^-. [Q_A^-] is the concentration of the reduced Q_A.

Table 1. The amplitude (A) and the halftime ($t_{1/2}$) of the fast (f) and slow (s) Q_A^- decay components in control, HCO_3^- depleted and HCO_3^- restored thylakoids of *Synechocystis* 6803 at pH 6.5 and pH 7.5. The parameters were calculated from the data after 3 actinic flashes. The flash frequency was 1 Hz.

Treatment	pH of reaction medium	A_f (%)	A_s (%)	$t_{1/2}$(f) (μs)	$t_{1/2}$(s) (ms)
Control	6.5	42 ± 1[a]	58 ± 1	403 ± 5	26 ± 1
	7.5	56 ± 2	44 ± 2	331 ± 3	21 ± 1
HCO_3^- depleted	6.5	14 ± 1	86 ± 2	433 ± 6	26 ± 1
	7.5	26 ± 1	74 ± 2	340 ± 3	33 ± 2
Restored	6.5	42 ± 1	58 ± 1	395 ± 4	25 ± 1
	7.5	57 ± 2	43 ± 2	328 ± 2	22 ± 1

[a] Standard deviations; number of experiments averaged, 4.

bicarbonate depletion must also impose a block on the electron transport from Q_A^- to the PQ pool.

The effect of bicarbonate on electron transport rates (O_2 evolution with DMQ as electron acceptor) in bicarbonate-depleted cells, following an

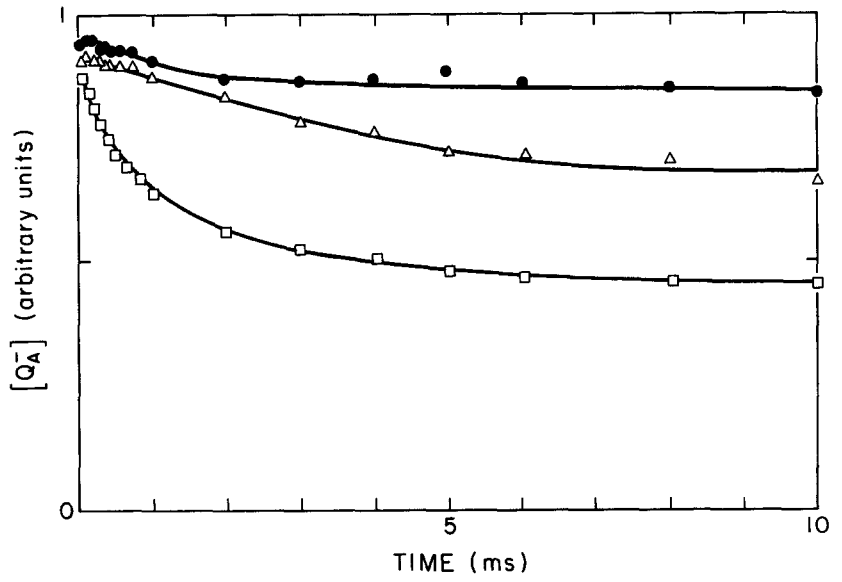

Fig. 2. Decay of Q_A^- after 3 actinic flashes in control (□), and DCMU-poisoned (●) thylakoids of *Synechocystis* 6803 at pH 6.5. For comparison with the bicarbonate-depletion effect, Q_A^- decay in a HCO_3^- depleted sample from Fig. 1A is also shown (△). The DCMU concentration was 5 μM. [Q_A^-] is the concentration of the reduced Q_A.

Table 2. Effect of bicarbonate on oxygen evolution in bicarbonate-depleted cells of *Synechocystis* 6803. The 2 ml reaction mixture contained 0.1 M sorbitol, 25 mM sodium formate, 10 mM NaCl, 5 mM $MgCl_2$, 20 mM NaH_2PO_4 (pH 6.5), and 0.1 μM gramicidin D. The reaction medium was adjusted to pH 6.5 after addition of 20 mM HCO_3^-. Depletion time was 4 h. Concentration of the inhibitor DBMIB was 20 μM. Artificial electron acceptors were DMQ (0.5 mM) and $K_3Fe(CN)_6$ (1 mM). Cells of *Synechocystis* 6803 containing 20 μg Chl*a* ml^{-1} were used.

Rate of oxygen evolution (μmol O_2 (mg Chl*a*)$^{-1}$ h^{-1})			Ratio $+HCO_3^-/-HCO_3^-$	
A $-HCO_3$ plus DMQ and $K_3Fe(CN)_6$	B $+20$ mM HCO_3^- minus DMQ and $K_3Fe(CN)_6$	C $+20$ mM HCO_3^- plus DMQ and $K_3Fe(CN)_6$	C–B	C–B/A
37.2 ± 4[a]	99.4 ± 14	263.3 ± 32	163.9 ± 17	4.4
		plus DBMIB		
35.7 ± 2	0.0 ± 1	150.3 ± 14	150.3 ± 7	4.2

[a] Standard deviations; number of experiments averaged, 4.

addition of 20 mM HCO_3^-, is shown in Table 2. After 4 h of bicarbonate depletion, the oxygen evolution rate was 37 μmol O_2 (mg Chl*a*)$^{-1}$ h^{-1}. The addition of 20 mM bicarbonate (pH adjusted to pH 6.5) stimulated the oxygen evolution rate to 263 μmol (mg Chl*a*)$^{-1}$ h^{-1}. For cells of *Synechocystis* 6803, bicarbonate was also a source of CO_2 for carbon reduction since an oxygen evolution rate of 99 μmol (mg Chl*a*)$^{-1}$ h^{-1} was observed in bicarbonate-recovered samples in the absence of artificial electron acceptors. The net stimulation (corrected for CO_2 fixation) in electron transport rate from water to DMQ by the addition of bicarbonate was 4.4 fold. To be sure that our conclusion was not affected by CO_2 fixation, we have done an experiment in which an inhibitor DBMIB, which blocks electron transport flow between plastoquinone pool and photosystem I (Trebst 1980), was used. In *Synechocystis* cells, a minimum of 20 μM DBMIB in the reaction mixture was found necessary to cause a practically complete blockage of electron transport. In chloroplasts of higher plants, however, lower concentration of DBMIB (0.2–1 μM) can inhibit electron flow. The difference may lie in the different accessibility of DBMIB in the reaction medium to the thylakoids in the whole cells and in the chloroplasts. Here, there was no electron flow after the addition of bicarbonate when there was no electron acceptor. In the presence of DBMIB and the electron acceptors DMQ and ferricyanide, addition of 20 mM bicarbonate stimulated the electron transport rate by 4.2 fold. Similar stimulative effect of bicarbonate was also observed in the

presence of an uncoupler S_{13}, a salicylanilide (data not shown), that also eliminates CO_2 fixation. Thus, the bicarbonate effect observed here is clearly unrelated to CO_2 fixation.

Concluding remarks

Our data in Table 2 show that 4 h bicarbonate depletion produces a significant 'bicarbonate effect' in intact *Synechocystis* 6803 cells indepedent of CO_2 fixation. Results presented in Figs. 1 and 2 and Tables 1 and 2 clearly show the 'bicarbonate effect' in both intact cells and thylakoids of *Synechocystis* 6803. However, van Rensen and Vermaas (1981), who had measured the Hill reactions in the thylakoids of a cyanobacterium *Anacystis nidulans*, found no bicarbonate effect. We do not know the reason for this negative result. Nevertheless, our data establishes the existence of a reversible bicarbonate effect in a cyanobacterium and, thus, makes it easier to unravel, in the future, the mechanism of the bicarbonate effect on the electron transport.

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