Comparison of Bicarbonate Effects on the Variable Chlorophyll *a* Fluorescence of CO_2-Depleted and Non-CO_2-Depleted Thylakoids in the Presence of Diuron

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Evidence is presented from chlorophyll *a* fluorescence transient data for two sites of bicarbonate (HCO_3^-*) action in photosystem II. Both the absence of HCO_3^- (HCO_3^--depleted thylakoids) and a high concentration of HCO_3^- (60 mM HCO_3^- added to non-depleted thylakoids) accelerate the variable chlorophyll *a* fluorescence rise in the presence of 10 µM diuron (DCMU). In non-HCO_3^--depleted thylakoids the effect is independent of the order in which HCO_3^- and DCMU are added, whereas in HCO_3^--depleted thylakoids, the effect is seen only when HCO_3^- is added before DCMU. We propose that the effect seen in HCO_3^--depleted thylakoids is indirectly due to the binding of HCO_3^- functionally near the site of DCMU binding, which is also where HCO_3^- exerts its major effect on electron transport between the primary quinone Q_A and the plastoquinone pool. We suggest that the smaller effect seen in non-HCO_3^--depleted thylakoids is due to the binding of HCO_3^- at a second, lower affinity site. Binding at this site appears to require light, in contrast to the higher affinity site, which is inhibited by light. Bathocuproine, an inhibitor of the H_2O-to-silicomolybdate partial reaction, is synergistic with HCO_3^- in its effect on the variable chlorophyll *a* fluorescence of non-HCO_3^--depleted thylakoids, and may bind heterotropically with HCO_3^-. Thus, this second site of HCO_3^- binding appears to be functionally near the bathocuproine binding site.

Introduction

Bicarbonate (HCO_3^-) appears to be required for photosystem II (PS II) electron transport. Depleting chloroplasts of HCO_3^-, in the presence of formate, causes a complete, or nearly complete, block between the secondary quinone Q_B and the plastoquinone (PQ) pool [1–3], as well as a deceleration of the rate of electron transfer from the primary quinone Q_A to Q_B [2, 4]. This effect is reversible, as electron transport can be almost completely restored by addition of HCO_3^-.

In DCMU-treated thylakoids that are depleted of HCO_3^-, the addition of HCO_3^- causes a deceleration of the chlorophyll *a* (Chl *a*) fluorescence rise during fluorescence induction measurements, indicating that when electron transport between Q_A and Q_B is blocked by DCMU, the absence of HCO_3^- further inhibits the oxidation of Q_A^- [3]. This inhibition may be on the back reaction of Q_A^- with $P680^+$ (see *e.g.* [3]).

In non-HCO_3^--depleted chloroplasts, HCO_3^- stimulates whole-chain electron transport with methyl viologen (MV) as acceptor, but it inhibits the PS II reduction of silicomolybdate (SiMo) [5]. It was suggested that SiMo accepts electrons, not directly from Q_A, as previously believed (*e.g.* [6]), but via a side chain from Q_A, which is blocked by HCO_3^- [5]. This observation suggests a HCO_3^- effect at a location other than the major effect at the Q_B-protein. We have investigated this hypothesis by comparing the effects of HCO_3^- on the fluorescence transients of DCMU-treated thylakoids that were or were not depleted of HCO_3^-. We present here evidence supporting the existence of two HCO_3^- binding sites in PS II.

Abbreviations: BSA, bovine serum albumin; Chl, chlorophyll; DCMU (diuron), 3-(3,4-dichlorophenyl)-1,1-dimethylurea; MV, methyl viologen; PS, photosystem; PQ, plastoquinone; Q_A, primary quinone electron acceptor of photosystem II; Q_B, secondary quinone electron acceptor.

* No distinction is made in this paper between HCO_3^- or CO_2 as the active species involved.

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Materials and Methods

Broken chloroplasts (thylakoids) were isolated at 7 C from hydroponically grown spinach by grinding the leaves for 10 s in cold isolation medium (20 mM HEPES, 15 mM NaCl, and 5 mM $MgCl_2$, adjusted to pH 7.5 at room temperature) along with 0.5% BSA (w/v) and 1 mM EDTA. The filtered homogenate

was pelleted by centrifugation at 3500 × *g* for 5 min, washed in cold isolation medium, recentrifuged, and finally resuspended in a minimum volume of isolation medium and 0.4 M sucrose. The final chlorophyll concentration was 3.5 mg/ml, as determined by the method of MacKinney [7]. The thylakoids were immediately frozen in 150 µl aliquots in liquid N_2, and stored there until use. HCO_3^--depletion was carried out according to methods previously described [3].

Fluorescence induction curves were measured at 685 nm on equipment already described [8]. The data were digitized with 8 bit precision by a Biomation waveform recorder (model 805) and stored on an LSI 11 minicomputer. If a gas stream was passed over the sample, it was first bubbled through H_2O to prevent evaporation of the sample, and the stream was kept to a minimum flow. Samples were illuminated for 30 s, and then dark-adapted 10 min prior to measurement.

In the experiments with non-HCO_3^--depleted thylakoids, 100 µl of saturated $NaHCO_3$ was added to the thylakoid suspension to make a final volume of 2 ml (60 mM $NaHCO_3$). This raised the pH considerably, unless a very high concentration of buffer was used. To get around this pH problem, the thylakoids were suspended in 50 mM sodium phosphate, pH 7.2, for those experiments not involving the addition of HCO_3^-. For experiments where 60 mM HCO_3^- was added, the thylakoids were suspended in 50 mM sodium phosphate, pH 6.6. Immediately upon addition of the HCO_3^-, which raised the pH to 7.2, a gentle gas stream at a partial pressure of CO_2 in equilibrium with a 60 mM HCO_3^- solution at pH 7.2 (22% CO_2 and 78% air) was passed over the sample. The pH of the solution at the end of each experiment was consistently 7.2.

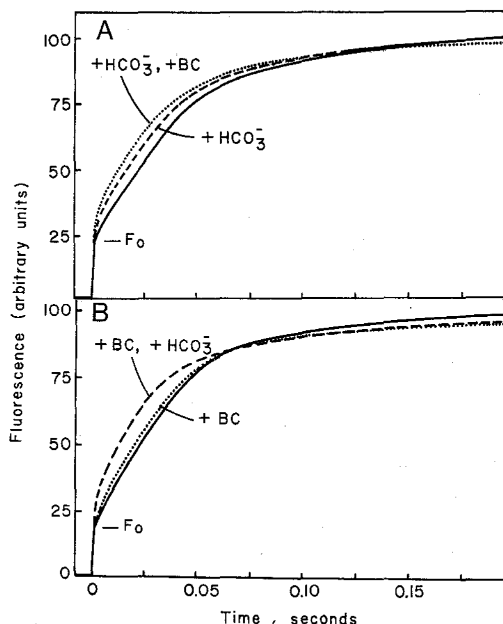

Fig. 1. Chlorophyll *a* fluorescence transients of non-HCO_3^--depleted thylakoids in the presence of DCMU, with and without HCO_3^- and bathocuproine (BC). [A]: dashed line is with 60 mM HCO_3^-; dotted line is with 60 mM HCO_3^-, followed by 60 µM BC. [B]: dashed line is with 60 µM BC, followed by 60 mM HCO_3^-; dotted line is with 60 µM BC. The solid line in both cases is with no additions other than 10 µM DCMU. All samples were illuminated 30 s, then dark adapted for 10 min prior to measurement. Changes in pH were controlled as described in Materials and Methods. The chlorophyll concentration was 25 µg/ml.

Results and Discussion

If a DCMU-insensitive side chain from Q_A is blocked by HCO_3^-, then HCO_3^- should cause a faster buildup of Q_A^- and an accelerated Chl *a* fluorescence rise in the presence of DCMU. When 60 mM HCO_3^- was added to non-HCO_3^--depleted thylakoids in the presence of DCMU, an accelerated Chl *a* fluorescence rise was indeed observed (Fig. 1). This effect appears to be specific for HCO_3^-, since raising the ionic strength by the addition of 60 mM NaCl, 60 mM $NaHCO_2$, or 60 mM Na_2SO_4 did not noticeably affect the fluorescence transient of DCMU-treated thylakoids (data not shown).

Bathocuproine (4,7-diphenyl-2,9-dimethyl-1,10-phenanthroline), like HCO_3^-, was observed to inhibit the H_2O-to-SiMo partial reaction, while accelerating electron flow from H_2O to MV [5]. We observed that bathocuproine accelerates the Chl *a* fluorescence rise in DCMU-treated thylakoids, as does HCO_3^- (Fig. 1). Interestingly, HCO_3^- and bathocuproine were observed to be synergistic in their effects on the fluorescence transient. In Fig. 1B, the effect of 60 µM bathocuproine is smaller than in Fig. 1A, where the bathocuproine was added after HCO_3^- addition. Similarly, 60 mM HCO_3^-, when added after bathocuproine, showed a larger effect than when it was added by itself. This synergism suggests a heterotropic binding of the two compounds.

This HCO_3^- effect appears to require light. When HCO_3^- was added in the dark, no effect on the fluo-

rescence transient was observed. The fluorescence rise was accelerated only after the thylakoids had been incubated with HCO_3^- briefly in the light (Fig. 2). In contrast, the restoration of the Hill activity in HCO_3^--depleted chloroplasts requires a dark incubation with HCO_3^- [9, 10].

In HCO_3^--depleted thylakoids, the addition of HCO_3^- causes a deceleration of the Chl *a* fluorescence rise in the presence of DCMU [3]. This is opposite of the effect observed in non-HCO_3^--depleted thylakoids (Fig. 1). Figure 3 shows the effect of 12 mM HCO_3^- on the Chl *a* fluorescence transient of HCO_3^--depleted thylakoids. When HCO_3^- was added before 10 μM DCMU, the fluorescence rise was slower than without HCO_3^- (Fig. 3A; [3]). However, when DCMU was added first, this HCO_3^- effect was not seen (Fig. 3B). This observation is in contrast to the effect seen in non-HCO_3^--depleted thylakoids, which is seen regardless of the order in which HCO_3^- and DCMU are added. Apparently, the binding of DCMU at the Q_B binding site (see *e.g.* [11]) prevented HCO_3^- from reaching its site of action in HCO_3^--depleted thylakoids [12], but not in the non-depleted samples. Therefore, we rule out the possibility that the two effects are due simply to the HCO_3^- concentrations being on opposite sides of a concentration optimum.

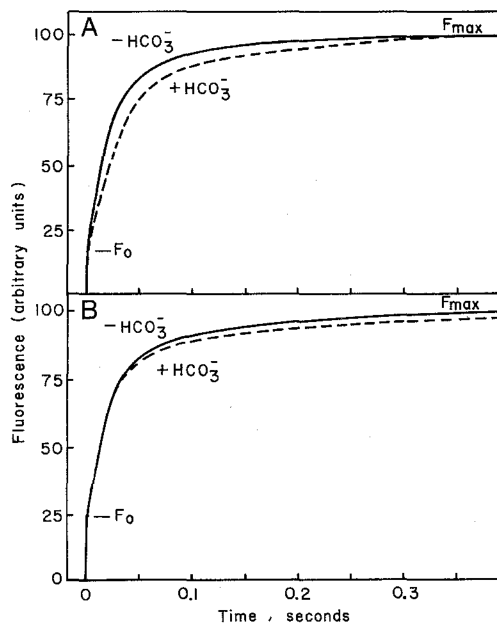

Fig. 3. Chlorophyll *a* fluorescence transients of HCO_3^--depleted thylakoids, when HCO_3^- was added before (A) or after (B) addition of DCMU. All samples contained 10 μM DCMU, either alone (solid line) or with 12 mM HCO_3^- (dashed line). The chlorophyll concentration was 25 μg/ml.

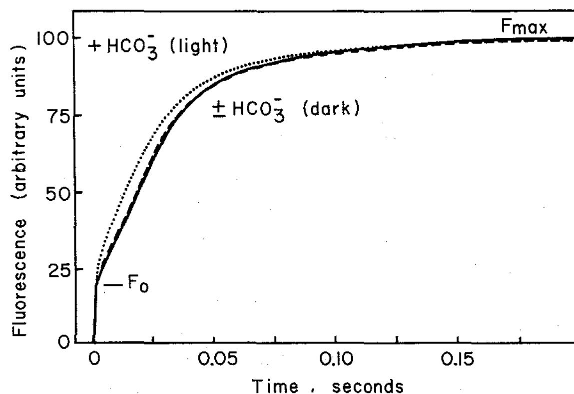

Fig. 2. Chlorophyll *a* fluorescence transients of non-HCO_3^--depleted thylakoids in the presence of DCMU, with (dotted line) and without (dashed line) a brief incubation in the light. All samples were illuminated 30 s, then dark adapted for 10 min prior to measurement. 60 mM HCO_3^- was added either before the illumination (dotted line) or during the subsequent dark period (dashed line). Control thylakoids were given 10 μM DCMU, but no HCO_3^- (solid line). Changes in pH were controlled as described in Materials and Methods. The chlorophyll concentration was 25 μg/ml.

Similarly, although light appears to be required for the binding of HCO_3^- to non-HCO_3^--depleted thylakoids (Fig. 2), in thylakoids that are depleted of HCO_3^-, the exposure to light seemed to prevent the binding of HCO_3^-. This was shown by illuminating the HCO_3^--depleted thylakoids throughout the incubation with HCO_3^-. When DCMU was added later in the light, followed by a dark adaptation, this HCO_3^- effect was not seen (data not shown). The result was the same as if DCMU had been added first (*i.e.* same as Fig. 3B).

These observations are readily explained by postulating two sites of HCO_3^- action. A high affinity site at the level of Q_B has been the subject of considerable study (for reviews, see [13–16]). Depleting HCO_3^- from this site inhibits electron transport between Q_A and PQ [1–4]. HCO_3^--depletion also reduces the binding of several DCMU-type herbicides [17–19], indicating a close interaction between the HCO_3^- site and the herbicide binding site. Light inhibits the binding of HCO_3^- to this site [9, 10], perhaps because the ratio of Q_B^-/Q_B is higher [20]. We propose that the effect of HCO_3^- on the Chl *a* fluorescence transient of HCO_3^--depleted

thylakoids (Fig. 3) is indirectly due to the binding of HCO_3^- at this high affinity site. We suggest that when electron transport from Q_A^- to Q_B is blocked by DCMU, HCO_3^- still allows some reoxidation of Q_A^-, either by an effect on the back reaction of Q_A^- with $P680^+$ [3], or perhaps by permitting electron flow *via* Q_2, which requires a higher DCMU concentration for a complete block [21].

A second HCO_3^- site, of much lower affinity, is functionally near the site of bathocuproine binding. The observation that HCO_3^- accelerates the fluorescence rise in DCMU-treated thylakoids indicates that Q_A is reduced faster at high HCO_3^- concentrations, which is consistent with the model of Barr and Crane [5; see Introduction, this paper], in which HCO_3^- inhibits a side chain from Q_A. This second site binds HCO_3^- preferentially in the light, whereas the binding of HCO_3^- at the high affinity site is inhibited by light. Furthermore, the order of addition of DCMU and HCO_3^- is crucial to the high-affinity site, but is of no consequence to the low-affinity site.

Acknowledgements

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