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BICARBONATE EFFECTS ON CHLOROPHYLL *a* FLUORESCENCE TRANSIENTS IN THE PRESENCE AND THE ABSENCE OF DIURON

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We investigated the effect of HCO_3^- addition to CO_2-depleted thylakoids by means of fluorescence techniques. (1) In the presence of diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea), the net reduction of the primary quinone-type electron acceptor (Q) of Photosystem (PS) II is about 2-times faster in the absence of HCO_3^- than in its presence, whether normal, heat-treated or NH_2OH-treated samples are used. This effect of HCO_3^- is, therefore, not on the O_2-evolving apparatus. It is, however, interpreted to be due to an influence of HCO_3^- on the kinetics of the reduction of Q, perhaps combined with an effect on the back reaction of Q^- with *P*-680$^+$, the oxidized form of the PS II reaction center chlorophyll *a*. (2) Fluorescence experiments in the absence of diuron indicate that the absence of HCO_3^- results in a complete block at the quinone level; the area over the fluorescence induction curve in the absence of HCO_3^- was found to be 2.2-times higher in the absence than in the presence of diuron, pointing to a complete block of BH_2 oxidation in the absence of HCO_3^-. (3) No change in the midpoint potential of Q is observed when HCO_3^- is added to CO_2-depleted membranes. HCO_3^- not only has a large (on/off) effect on the reoxidation of BH_2, but also a smaller effect between *P*-680 and Q. We propose that HCO_3^- binding to its specific site in the thylakoid membrane results in a conformational change, allowing normal electron transport between the two photosystems.

Introduction

Warburg and Krippahl [1,2] observed that the Hill reaction with quinone or $Fe(CN)_6^{3-}$ as electron acceptor is dependent on HCO_3^-. In the absence of HCO_3^-, a low Hill reaction rate is observed, but the addition of HCO_3^- results in an enhancement of this activity. (Since it is not yet certain whether CO_2, HCO_3^- or CO_3^{2-} is responsible for this effect, although some evidence points to HCO_3^- as the 'binding species' [3], we will use 'HCO_3^-*' to designate the species that binds to a specific site in the thylakoid membrane exerting its stimulatory effect on photosynthetic electron transport.) A major site of action of HCO_3^-* has been shown to be between Q, the first quinone-type PS II acceptor, and the plastoquinone pool [4]. In the absence of HCO_3^-*, the oxidation of Q^- by B, the quinone-type two-electron gate, is slowed down [5–8], and the rate of B^{2-} oxidation by plastoquinone is also decreased dramatically [6,7]. Although an effect of HCO_3^-* on the oxygen-evolving system has been postulated [9–14], most of the evidence obtained thus far points to an HCO_3^-* action on the electron-acceptor side of PS

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Abbreviations: PS, photosystem; Chl, chlorophyll; diuron, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU).

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II [4,15–18]. Absence of HCO_3^-* not only inhibits electron transport from Q^- to plastoquinone, but it also seems to block the diuron-insensitive electron transport from Q^- to C400 [19], a postulated auxiliary PS II acceptor, and to accelerate the Q^- accumulation in the presence of diuron upon illumination as measured by the Chl *a* fluorescence induction curve [20]. We confirm the faster Q^- accumulation in the absence of HCO_3^-* and in the presence of diuron, and show that this effect is located between the PS II reaction center, *P*-680, and Q, and not on the electron-donor side of PS II as was suggested before [20].

The Chl *a* fluorescence induction curve, monitoring the rate of the reduction of Q (Q is a fluorescence quencher, whereas Q^- is not [21]), has been shown to consist of a sigmoidal fast phase and a slow phase in the presence of diuron [22–25]. This biphasic behavior was interpreted to be the result of a heterogeneity in the environment of PS II reaction centers: certain reaction centers were postulated to be in contact with adjacent PS II reaction centers and with a large number of antenna chlorophyll molecules (α-centers), whereas other PS II reaction centers (β-centers) were to have a small absorption cross-section and lack contact with neighboring PS II reaction centers [25,26]. In this paper, we have also analyzed the fluorescence induction curve in CO_2-depleted thylakoids to see if the absence of HCO_3^-* results in a change in the ratio of Q related to α-centers (Q_α) and Q_β.

Furthermore, we show here that in thylakoids thoroughly depleted of CO_2, the rise kinetics of fluorescence induction are best explained by a complete block between Q and plastoquinone. In the absence of HCO_3^-* the area over the fluorescence induction curve is a little more than twice as large without than with diuron, and we interpret this as indicating a complete block between B^{2-} and plastoquinone.

Materials and Methods

Chloroplast isolation and CO_2 depletion

Chloroplasts were isolated from peas (*Pisum sativum*), grown at 20°C, and were broken as described elsewhere [27]. Broken chloroplasts (thylakoids) were stored at −60°C. For CO_2 depletion, if required, thylakoids were thawed and suspended in CO_2-free depletion medium under an N_2 atmosphere to a chlorophyll concentration of approx. 30 μg/ml. CO_2-free media and tubes were obtained by bubbling and flushing, respectively, with N_2 gas led through a column containing ascarite and soda-lime. The CO_2-depletion medium consisted of 50 mM sodium phosphate, 100 mM HCO_2Na, 100 mM NaCl and 5 mM $MgCl_2$, pH 5.3 (HCO_2^- is supposed to bind to the same site as HCO_3^-* [3,4] (Good, N., personal communication)). Thylakoids were incubated for 5 min in the dark at 0°C and pelleted (5 min at 1000 × *g*). If thoroughly CO_2-depleted thylakoids were required, the pellets were washed again in the pH 5.3 medium (twice-washed thylakoids). The thylakoid pellet was resuspended in a CO_2-free medium consisting of 50 mM sodium phosphate, 100 mM HCO_2Na, 100 mM NaCl and 5 mM $MgCl_2$, pH 6.5. Extreme care was taken that the thylakoid suspension was not exposed to a CO_2-containing atmosphere. After this treatment, the $Fe(CN)_6^{3-}$ or 2,6-dichlorophenolindophenol Hill reaction was low, whereas after the addition of 10 mM $NaHCO_3$ an at least 8-fold increase in the Hill reaction rate was observed, indicating that this mild CO_2 depletion, a modification of the method used by Stemler [28], works just as well as the rather rough methods of CO_2 depletion used before [3,4,20]. In twice-washed thylakoids, the Hill reaction rate before HCO_3^- addition was negligible (less than 3 μmol O_2/mg Chl per h).

We note that even after this mild method of CO_2 depletion, the Hill reaction rates after the addition of saturating HCO_3^- quantities (10 mM) were lower than those in untreated chloroplasts (approx. 50% of the original rates). This shows that a considerable irreversible inactivation occurs due to the depletion procedure. The same irreversible inactivation was found if thylakoids were washed in CO_2-sufficient medium containing 50 mM sodium phosphate, 200 mM NaCl and 5 mM $MgCl_2$ (pH 5.3) (Vermaas, W. and Govindjee, unpublished results), indicating that this inactivation is not due to CO_2 depletion but to the treatment at low pH and high salt concentration. The site of this irreversible effect, mainly due to the low pH treatment, may be electron donation to *P*-680$^+$ (cf. Ref. 29). However, to achieve good

CO_2 depletion, such conditions were found to be necessary. Irreversible loss of some electron-transport activity seems to be unavoidable in the CO_2-depletion procedure. (However, see also Ref. 14.)

Fluorescence measurements

Chl *a* fluorescence induction measurements were made with an instrument slightly modified from that of Munday and Govindjee [30], using chloroplasts that were dark adapted for at least 20 min. Actinic light was provided by a tungsten lamp; this light passed a water bath and Corning glass filters 4-76 and 3-73 before it hit the sample. The intensity of the actinic light (400–700 nm) was measured with an LI 190S Quantum sensor (Lambda Instuments Corp.). The photomultiplier (EMI 9558 B) was shielded from the actinic light by a Corning 2-64 filter and Bausch and Lomb monochromator (λ 685 nm, band width 12 nm). The fluorescence signal was amplified and recorded on a Tektronix 502 oscilloscope equipped with a polaroid camera. When relatively slow (greater than 1 s) transients were measured, the amplified photomultiplier signal was monitored with an Esterline Angus recorder. The area over the fluorescence induction curve as a function of time was determined as described by Melis and Homann [25].

In order to avoid 'artifacts' due to differences in dark-adaptation rates in CO_2-depleted chloroplasts in the absence and presence of HCO_3^- [20], addition, if necessary, of 10 mM $NaHCO_3$ to CO_2-depleted thylakoid samples was made in the dark 1 min before the fluorescence measurement. Therefore, thylakoids were always dark adapted without added HCO_3^-.

CO_2-depletion and fluorescence measurements, made to elucidate the site of HCO_3^-* action between Q and plastoquinone, were performed under CO_2-free air instead of CO_2-free N_2 because it was suggested [20] that anaerobic conditions might decrease the dark-adaptation (oxidation) rate of electron acceptors, for instance the plastoquinone pool.

Redox titrations

Fluorescence induction measurements as a function of redox potential were performed in the apparatus described above; the sample holder used for the measurements was connected, via a pump, to a large reservoir containing a dark-adapted thylakoid suspension. After flushing the sample holder and the O_2-impermeable tubing between sample holder and reservoir with a large volume (approx. 50 ml) of the thylakoid suspension equlibrated at a certain redox potential, 3 ml of the suspension were allowed to remain in the sample holder; this sample was used for the fluorescence induction measurements. After the measurement, the illuminated sample was removed from the sample holder by a syringe and discarded. Both the sample holder and the reservoir were kept free of O_2 and CO_2 by flushing with purified N_2. Redox mediators (duroquinone, 9,10-anthraquinone-1,5-disulfonate and benzyl viologen) were added to the reservoir to a final concentration of 10 μM. Reductive titrations were performed by adding small quantities of a freshly prepared dithionite solution to the reservoir. The addition of dithionite did not decrease the pH by more than 0.2 units. The redox potential was measured by a combination of a calomel and a platinum electrode (Radiometer type K401 and P101, respectively), connected to a Radiometer PHM 63 digital pH meter. The redox potential of the calomel electrode relative to the standard hydrogen electrode was accepted to be +244 mV. The electrodes were regularly checked using a mixture of 10 mM $K_4Fe(CN)_6$/10 mM $K_3Fe(CN)_6$ in 100 mM KCl. The redox potential for this couple was taken to be +436 mV [31].

Results

The influence of HCO_3^- on the Chl a fluorescence induction kinetics in the presence of diuron*

Fig. 1A shows that the absence of HCO_3^-* leads to a faster accumulation of Q^- in thylakoids in the presence of diuron which is in agreement with earlier observations [20]. The initial and the maximal fluorescence yields are the same in the presence and the absence of HCO_3^-*, indicating that HCO_3^-* does not influence the number of active PS II reaction centers. In the presence of diuron the area over the fluorescence rise curve monitors the concentration of Q [32]. We checked to see if the faster rate of reduction of Q involved a change in the biphasic behavior of the fluores-

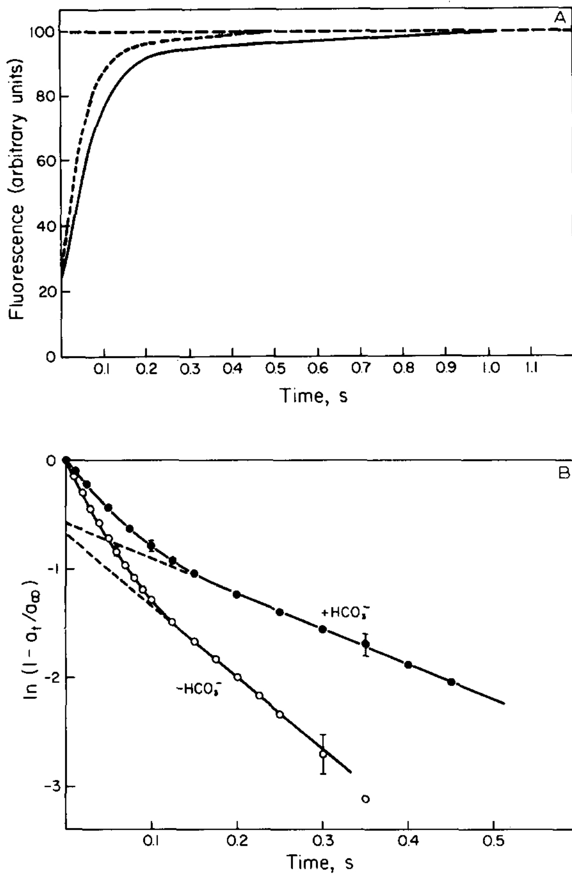

Fig. 1. Chl *a* fluorescence induction curves of CO_2-depleted thylakoids with 10 μM diuron in the presence (———) or absence (- - - - - -) of 10 mM HCO_3^- (A). The natural logarithm of the relative complementary area over the induction curve at time t ($\ln(1-a_t/a_\infty)$) is calculated at various times for the induction curves with (●) and without (○) HCO_3^- addition and is plotted in B. The light intensity was 15 $\mu E/m^2$ per s; the chlorophyll concentration was 15 μg/ml.

cence induction curve. The relative concentration of Q in its oxidized form at time t was calculated [25] from the ratio $(1 - a_t/a_\infty)$, in which a_t is the area over the fluorescence inducation curve at time t and a_∞ is the area at infinite time (all Q reduced). Fig. 1B indicates that the amplitude of the slow phase is somewhat larger after the addition of HCO_3^- than without HCO_3^- addition; also, the rate constants of both the slow and fast phases are higher in the absence than in the presence of HCO_3^-. These differences were observed consistently; the 'tail' in the induction curve was always more pronounced in $+HCO_3^-$ than in $-HCO_3^-$* curves. A possible explanation for this phenomenon is that in the absence of HCO_3^-* more PS II reaction centers are 'connected' to each other than in its presence.

To locate this HCO_3^-* effect in the presence of diuron more precisely, experiments were performed with CO_2-depleted thylakoids in which the oxygen-evolving system had been inactivated by mild heat treatment (50°C, 75 s) [33]. Fig. 2A shows that Q reduction in heat-treated CO_2-depleted thylakoids in the presence of an artificial donor (100 μM phenylenediamine + 2 mM ascorbate) is still influenced by HCO_3^- addition, indicating that the site of this HCO_3^-* action is not the oxygen-evolving system. Tris treatment, which

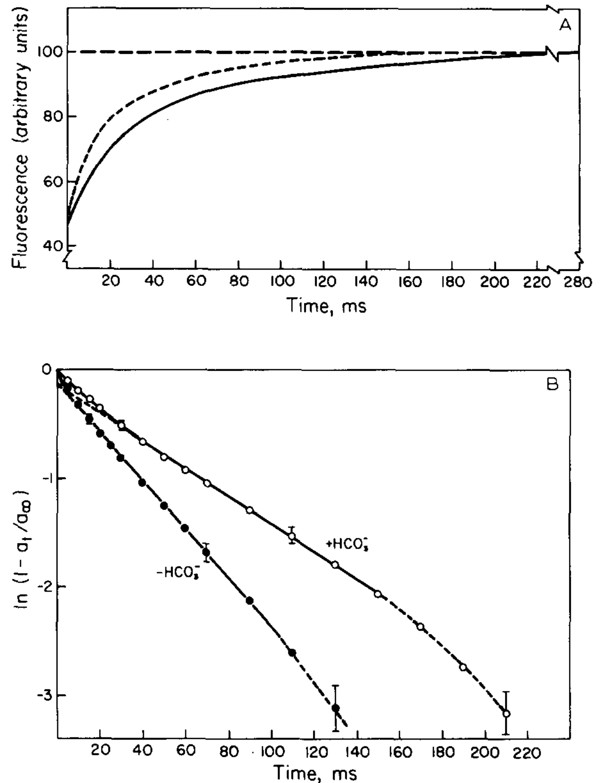

Fig. 2. Chl *a* fluorescence induction curves of heat-treated CO_2-depleted thylakoids with 10 μM diuron in the presence (—) or absence (- - - - - -) of 10 mM HCO_3^- (A). The natural logarithm of the complementary area over the induction curve is calculated at various times with (●) and without (○) HCO_3^- addition and is plotted in B. Heat treatment: 75 s at 50°C; electron donation by 100 μM phenylenediamine + 2 mM ascorbate. The light intensity was 15 $\mu E/m^2$ per s; the chlorophyll concentration was 15 μg/ml.

also inactivates the oxygen-evolving system, leads to qualitatively identical results as obtained by heat treatment. After heat treatment the ratio of variable to total fluorescence yield is lowered compared to that of the control, indicating perhaps that certain reaction centers are inactivated resulting in relatively less open traps. This might also explain the faster fluorescence induction (Q reduction) in heat-treated (Fig. 2A) as compared to untreated thylakoids with an intact oxygen-evolving system (Fig. 1A). To observe the kinetics of the area growth, a $\ln(1-a_t/a_\infty)$ vs. time plot was constructed. As can be seen from Fig. 2B, Q reduction under these conditions became nearly monophasic. The slow phase seems to disappear. This is in agreement with the observation by Thielen and Van Gorkom [34] that in Tris-washed chloroplasts no slow phase is detectable.

It is known [35] that treatment with relatively high concentrations of NH_2OH (approx. 1 mM) inhibits the reduction of P-680$^+$ by its physiological donor, Z; P-680$^+$ is, under those conditions, suggested to be reduced by NH_2OH via a donor D [34]. To determine whether HCO_3^-* in the presence of diuron acts on Z, the effect of HCO_3^-* addition on the fluorescence induction kinetics in CO_2-depleted thylakoids was measured after pretreatment with 1 mM NH_2OH for 5 min at room temperature. The area over the induction curve of thylakoids in the absence of HCO_3^- was approx. 1.5-times smaller than that in the presence of HCO_3^-; the same factor is found in CO_2-depleted 'control' thylakoids, i.e., not treated with NH_2OH (data not shown). This indicates that the observed HCO_3^-* action in the presence of diuron is not located at the physiological PS II donor Z.

The midpoint potential of Q

Since most reactions involving Q^- reoxidation (i.e., by B, and, probably, C400 and P-680$^+$) seem to be slowed down or blocked in the absence of HCO_3^-*, it is feasible that the Q/Q^- midpoint potential is influenced by HCO_3^-*. To check this possibility a redox titration of Q was performed in the region from −20 to −220 mV, without diuron, in the presence and absence of HCO_3^- (Fig. 3). (We show only the reductive titration, since the Q^- reoxidation under anaerobic conditions was sluggish and incomplete (cf. Ref. 36).)

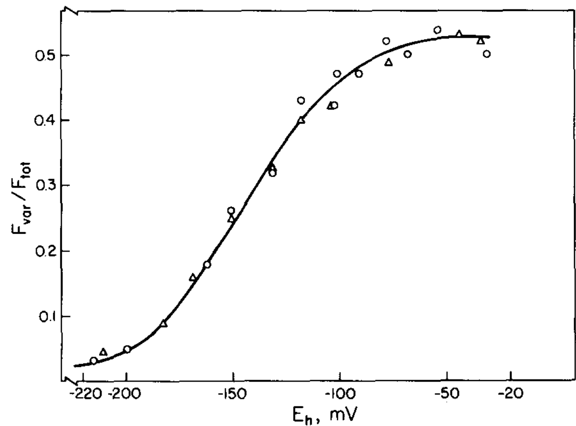

Fig. 3. Titration of the variable Chl *a* fluorescence as a function of redox potential in CO_2-depleted thylakoids in the presence (Δ) or absence (○) of 10 mM HCO_3^-. Redox mediators were duroquinone ($E_{m,7}$ = +5 mV), 9,10-anthraquinone-1,5-disulfonate ($E_{m,7}$ = −174 mV) and benzyl viologen ($E_{m,7}$ = −311 mV), all present at 10 μM. The chlorophyll concentration was 12 μg/ml.

The variable fluorescence disappears almost completely at −220 mV and a midpoint potential E_m of −145 mV (n = 1) (pH 6.5), both in the absence and in the presence of HCO_3^-*, is observed. The changes in variable fluorescence as a function of redox potential are not caused by the redox mediators used, since, for instance, omission of 9,10-anthraquinone-1,5-disulfonate ($E_{m,7}$ = −174 mV) does not change the observed pattern (data not shown). The data presented in Fig. 3 indicate that the apparent E_m of Q/Q^- does not change upon HCO_3^- addition to CO_2-depleted thylakoids.

The main site of HCO_3^- action*

In the absence of HCO_3^-*, the reoxidation of B^{2-} has been suggested to be influenced more than the reoxidation of Q^- [6,7]. However, there is no unambiguous proof for such a statement. It is also not known whether or not the absence of HCO_3^-* in a certain photosynthetic chain blocks electron transport completely in that chain (e.g., compare Refs. 7 and 37). To elucidate if the absence of HCO_3^-* results in a complete block of electron transfer at the quinone level or only in a slowing down of electron transport, we measured the fluorescence transient in thoroughly CO_2-depleted (twice-washed) thylakoids with and without HCO_3^- addition. In the absence of

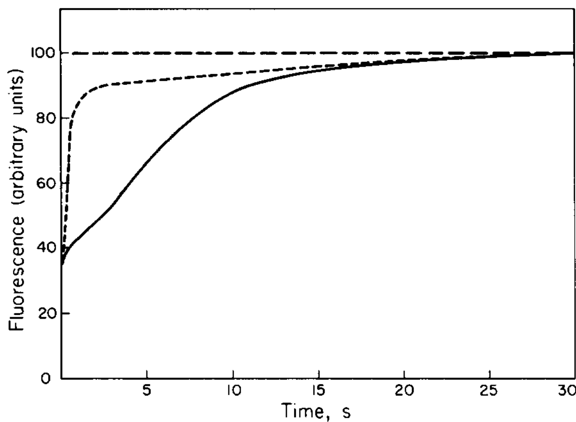

Fig. 4. Chl *a* fluorescence induction curves of CO_2-depleted thylakoids without diuron in the presence (———) and absence (- - - - - -) of 10 mM HCO_3^-. The fluorescence intensity at 30 s has been arbitrarily taken as 100. The light intensity was approx. 7 $\mu E/m^2$ per s; the chlorophyll concentration was 15 $\mu g/ml$.

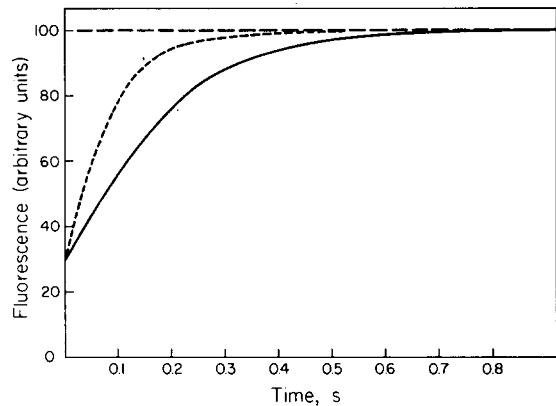

Fig. 5. Chl *a* fluorescence induction curves of CO_2-depleted chloroplasts without (———) and with (- - - - - -) the addition of 10 μM diuron. The light intensity was 11 $\mu E/m^2$ per s; the chlorophyll concentration was 15 $\mu g/ml$.

HCO_3^-*, the fluorescence level increases fast (similar but not identical to control samples with diuron) to a high level (indicating a rapid accumulation of Q^-) followed by a very slow increase (Fig. 4). After the addition of HCO_3^- a 'normal' fluorescence transient is observed indicating uninhibited electron flow. The tail of this fluorescence induction curve in thylakoids in the presence of HCO_3^-* shows the same very slow increase as observed in the absence of HCO_3^-* (Fig. 4). Therefore, we suggest that most of this very slow increase in fluorescence yield does not reflect a change in [Q^-], but is caused by secondary factors influencing the fluorescence yield. Thus, CO_2 depletion seems to cause a 'complete' block between Q and plastoquinone.

Jursinic and Stemler [8] report that the Q^- decay in CO_2-depleted thylakoids shows a relatively large amount of a very slow component ($t_{1/2}$ = 1–2 s). Therefore, we determined whether the major influence of CO_2 depletion on fluorescence induction is at the Q/B or at the B/plastoquinone level. Since the area over the fluorescence induction curve is assumed to be proportional to the number of PS II turnovers [38], we compared the areas over the induction curves in thoroughly CO_2-depleted thylakoids in the absence and presence of diuron (Fig. 5). Here, the ratio (area − diuron)/(area + diuron) is 2.2. This indicates that, in the absence of HCO_3^-* and diuron, Q and two-electron gate B, which is partly in the semiquinone form B^- after dark adaptation, can be reduced, whereas in the presence of diuron only Q can be reduced. Therefore, absence of HCO_3^-* results in a complete inhibition of B^{2-} oxidation by plastoquinone.

It should be noted that the thylakoids used in this experiment do not show an appreciable slow phase in the fluorescence induction curve in the presence of diuron (cf. Figs. 1A and 5). A large variability in the ratio of the amplitude of the slow and fast phase appears to exist between different thylakoid preparations.

Discussion

The rise of the Chl *a* fluorescence induction curve in the presence of diuron is faster in the absence of HCO_3^-* than in its presence (Figs. 1 and 2); this HCO_3^-* effect seems to be located at sites involving *P*-680, pheophytin or Q, since NH_2OH incubation, known to block electron transport from the physiological donor Z to *P*-680 [35], does not overcome this effect of HCO_3^-*. Furthermore, this approx. 2-fold effect is present in heat-treated and Tris-washed thylakoids that have lost the oxygen-evolving reactions. Therefore, it is not possible that this HCO_3^-* effect is directly

involved in oxygen evolution, although other effects of HCO_3^-* on oxygen-evolution steps have been suggested by other researchers [10–14]. The observation that the approx. 2-fold difference in fluorescence induction is still present after NH_2OH treatment rules out the possibility that a difference in the rate of the back reaction between Q^- and P-680^+ in the presence and absence of HCO_3^-* is the only cause of the HCO_3^-* effect on fluorescence induction in the presence of diuron: NH_2OH is known to slow down that back reaction (e.g. see Ref. 39). Furthermore, the microenvironment of Q does not seem to be sensitive to HCO_3^-*: the apparent E_m of Q/QH is independent of HCO_3^-*. Although we find one phase in the redox titration of our samples, Q is normally found to be reduced in a biphasic fashion: one component of Q (Q_L) titrates at $E_m \approx -200$ mV (pH 7), the other (Q_H) at approx. -30 mV (pH 7) [40,41]. Assuming a pH dependence of -60 mV/pH unit for Q_L [36], i.e., considering a protonation of Q_L^- under these conditions, Q_L is expected to titrate at about -170 mV at pH 6.5, which is rather close to the E_m of -145 mV (pH 6.5) reported here. We do not speculate at this moment on to the reason why no Q_H is detected in our samples.

Although the exact origin of the HCO_3^-* effect in the presence of diuron has not been found yet, HCO_3^-* has an effect, additional to the large one between BH_2 and plastoquinone, on the kinetics of Q^- formation.

In thylakoids without diuron, the absence of HCO_3^-* appears to result in a complete block of the $BH_2 \rightarrow$ plastoquinone reaction (Figs. 4 and 5): in the absence of HCO_3^-*, the ratio of the areas over the fluorescence induction curve in the absence and presence of diuron is 2.2 (Fig. 5). Thus, the plastoquinone pool is not filled, only the Q and B pools are filled. The somewhat low ratio of the areas (−diuron)/(+diuron) (2.75 would be expected when 75% of B is in the fully oxidized form after dark adaptation) may be due to the increased amplitude of the slow component of the Q^- decay [8]: in the time of the experiment not all Q may have reduced B completely, resulting in a somewhat lower area in the absence of diuron on the time scale used, and thus, in a lower ratio of the areas.

At the relatively low light intensity used, it takes approx. 5 s to fill half of the plastoquinone pool in the presence of HCO_3^-* (at 5 s the area over the fluorescence curve is about half of the total area of the curve; Fig. 4). If we assume that the photochemically active plastoquinone pool consists of approx. 6 plastoquinone molecules per PS II [42] (12 electron equivalents), then once in about 5/6 s (about 0.8 s) an electron is transported into the plastoquinone pool. In the absence of HCO_3^-*, the Chl a fluorescence rises to its almost maximal value within about 2 s (Fig. 4), suggesting a very slow (much greater than 0.8 s; it could be of the order of several seconds or, perhaps, minutes) leakage of electrons to the plastoquinone pool; this is much slower than the half-time of approx. 200 ms suggested earlier from experiments in repetitive flashing light [6,7].

The proposed site of a complete blockage, by the absence of HCO_3^-*, on the $B^{2-} \rightarrow$ plastoquinone reaction (Figs. 4 and 5) is rather unique although a total inhibition of electron flow in membranes having no bound HCO_3^-* molecule had been suggested earlier [37]. Most inhibitors (e.g., diuron, atrazine and ioxynil) block the Q^- oxidation, whereas others (2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone and the dinitrophenyl ether of iodonitrothymol) block the PQH_2 oxidation. If we assume that under normal conditions an exchange between B and plastoquinone is possible, then a block of the BH_2 oxidation might indicate that B is insulated from the plastoquinone pool in the absence of HCO_3^-*.

A working hypothesis that emerges now is that the absence of HCO_3^-* changes the conformation in the Q/B region such that (1) Q becomes 'separated' from B, and (2) B becomes 'insulated' from the plastoquinone pool. A possibility to explain this 'insulation' is that, in the absence of HCO_3^-*, BH_2 has a high affinity for its binding site on the B-binding protein and, thus, is not released and cannot transfer its electrons to the next acceptor. The proposed conformational change caused by binding or release of HCO_3^-* is very specific for HCO_3^-*: we have found (unpublished results) that neither HCO_3^- analogs like HSO_3^- or HPO_3^{2-} nor CO_2 analogs like CS_2 are able to restore the Hill reaction rate in CO_2-depleted chloroplasts. It is already known that HCO_2^- cannot restore electron transport in CO_2-depleted chloroplasts although it

is supposed to bind to the HCO_3^- *-binding site [3,4]. Therefore, we propose that the 'bicarbonate action' is extremely specific for HCO_3^- * (also, see Ref. 43) and that, perhaps, a binding of different groups of the HCO_3^- * molecule (e.g., C–OH or $C–O^-$) to different sites at the same time is required for the recovery of electron transport (see also Ref. 3).

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