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Electron transfer through the quinone acceptor complex of Photosystem II in bicarbonate-depleted spinach thylakoid membranes as a function of actinic flash number and frequency

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In this paper four major points with respect to HCO_3^--reversible inhibition in spinach thylakoid membranes, depleted of HCO_3^- in the presence of inhibitory anions, are established. (1) The oxidation of Q_A^- (Q_A is the primary quinone acceptor of Photosystem II) by Q_B (Q_B is the secondary quinone acceptor of Photosystem II) or Q_B^-, following one or two actinic flashes, respectively, exhibits a smaller t_{50} (time at which $[Q_A^-]$ is 50% of maximum $[Q_A^-]$) after a flash at pH 7.5 than at pH 6.5. (2) The characteristic oscillations, due to differential rates of Q_A^- oxidation by Q_B or Q_B^-, observed in the fluorescence flash pattern, generated by assaying the chlorophyll *a* fluorescence at specific times after an actinic flash and plotting these data as a function of flash number, are lost (i.e., the turnover of two electron gate is hampered). (3) At 1 Hz, the slowest oxidation of Q_A^-, as indicated by t_{50} values, depends on both the pH and flash number: at pH 6.5, t_{50} for Q_A^- decay reaches a maximum value after only three flashes (one turnover of the two-electron gate), whereas at pH 7.5, the t_{50} is increased further until after five flashes (two turnovers of the two-electron gate). (4) The t_{50} values of Q_A^- oxidation also depend on the actinic flash frequency: at 5 Hz, $[Q_A^-]$ reaches its maximum after flash 5 even at pH 6.5. The increase observed in the t_{50} values of Q_A^- oxidation in treated membranes is accompanied by the presence of slow components of Q_A^- oxidation in the 0.1–10 s range which can achieve an amplitude of more than 70%. These components are suggested to be related to protonation steps involved in the quinone acceptor complex of Photosystem II and support the conclusion that the rate-limiting step in electron transfer in HCO_3^--depleted thylakoids may be the protonation of Q_B^- and possibly Q_B^{2-}. A working hypothesis is presented that explains the flash, frequency and pH dependence of Q_A^- decay observed in this paper.

Introduction

Bicarbonate (or CO_2) was shown by Warburg and Krippahl [1] to stimulate electron transport during the Hill reaction (for reviews on this subject, see Refs. 2–6). This phenomenon has been referred to as the bicarbonate (HCO_3^-) effect, and it has been shown that HCO_3^-, and not CO_2 or CO_3^{2-}, is the active species involved [7]. The major

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Abbreviations: Chl, chlorophyll; t_{50}, time at which the $[Q_A^-]$ is 50% of the maximum $[Q_A^-]$ after a flash; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Mes, 4-morpholineethanesulphonic acid.

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rate-limiting step in HCO_3^--depleted or -treated membranes is at the level of the passage of electrons through the plastoquinone acceptors, Q_A and Q_B, that operate as a two-electron gate [8–11]. Q_A is the primary quinone acceptor and functions as an obligate one electron accepting species; and Q_B is the secondary quinone acceptor and is able to be doubly reduced to plastoquinol by two successive turnovers of the reaction center [12,13]. The secondary quinone acceptor is, therefore, capable of oxidizing Q_A^- in its plastoquinone (Q_B) as well as its plastosemiquinone (Q_B^-) forms [14,15].

The principal arguments to support the site of action of HCO_3^- on the electron acceptor side of Photosystem II (PS II) are: (1) the reoxidation of Q_A^-, as measured by the chlorophyll (Chl) *a* fluorescence yield decay [9,16] or by the absorbance change at 320 nm [10,17], is stimulated to be 10–20-fold faster by the HCO_3^- addition to HCO_3^--depeleted membranes; (2) in PS II particles, a light- and chemically induced EPR signal ($g = 1.82$), attributed to the Q_A^--Fe^{2+} complex, is reversibly increased in amplitude by a factor of about 10 by HCO_3^--depletion [18]; (3) the Chl *a* fluorescence yield [8] and thermoluminesence [11] after a series of single flashes of light suggest a dramatic slowing down of electron flow after the third and subsequent flashes following HCO_3^--depletion, which is totally reversed upon HCO_3^--addition; and (4) HCO_3^- depletion causes a several-fold change in the affinity of the binding of ^{14}C-atrazine or ^{14}C-ioxynil, that bind in the Q_B-apoprotein region [19,20].

Two principal mechanisms have been proposed to explain the action of HCO_3^- on electron transport through the plastoquinone acceptors of PS II. These are: (1) that bound HCO_3^- brings about a conformational change in the quinone acceptor complex facilitating electron transfer (e.g., Refs. 11, 19 and 21); and (2) that HCO_3^- is involved in the protonation steps associated with Q_B reduction to Q_B^{2-} ($2H^+$) (e.g., Refs. 6 and 22). Both of these hypotheses have been advanced to explain the result that the inhibition due to HCO_3^--depletion, in the presence of formate or nitrite [23,24], attains a maximum level after two turnovers of the reaction center [8,10,11,16]. The pH of samples in these experiments was either 6.5 or 6.8. Since the inhibited state after three actinic flashes was characterized by a high level of Chl*a* variable fluorescence, the inhibited reaction center was suggested to be locked in the state $Q_A^-Q_B^{2-}$ [8,11,21]. The conformational hypothesis then required that the rearrangement of the quinone acceptor complex prevented the release of Q_B^{2-} to the plastoquinol pool. The protonation hypothesis implied that the Q_B^{2-} could not be released to the plastoquinone pool until the state $Q_B^{2-}(2H^+)$ was formed.

The experiments in this paper were designed to understand further the mechanism of HCO_3^- action at the quinone acceptor complex, specifically in the electron transport from Q_A^- to Q_B or Q_B^-. To do this, we examined the HCO_3^--reversible inhibition of Q_A^- oxidation, measured by chlorophyll *a* fluorescence yield changes, as a function of flash number, flash frequency and pH. A preliminary observation on flash-number dependence has been presented, in an abstract form, in the proceedings of a conference [25]. On the basis of results reported here, we propose a working hypothesis in which HCO_3^--depletion or the presence of inhibitory anions, induces a conformational change in the quinone acceptor complex so as to inhibit the protonation of Q_B^- and possibly Q_B^{2-}.

Materials and Methods

The spinach used for this study was obtained from a commercial source originating from widely different locations over a period of 18 months. All data shown are typical results obtained from at least three separate bushels of spinach obtained on an approximately weekly basis.

Thylakoids membranes were isolated from spinach leaves by first grinding leaf segments in a medium containing 400 mM sorbitol, 50 mM NaCl, 1 mM EDTA (ethylenediaminetetraacetic acid) and 50 mM Hepes (pH 7.8) for 5 s in a Waring blendor. The resultant homogenate was filtered through 6 and then 12 layers of cheesecloth. The filtrate was then spun at $5000 \times g$ for 1 min, including the acceleration time, to remove any remaining debris. The filtrate of this step was then spun at $5000 \times g$ for 10 min. After discarding the last supernatant the pellet was resuspended and osmotically shocked in a medium containing 50 mM NaCl, 5 mM $MgCl_2$ and 10 mM Hepes

(pH 7.8). The suspension was then spun again at 5000 × g for 10 min and resuspended at approx. 2 mM Chl in 400 mM sorbitol, 15 mM NaCl, 5 mM $MgCl_2$ and 20 mM Hepes (pH 7.8). Chlorophyll concentrations were determined in micromolar units (for details and references, see Graan and Ort [26]). All isolation procedures were carried out at 0–4°C. The bicarbonate-depletion procedure (see below) was performed immediately following the isolation of the thylakoid membranes.

Bicarbonate-depleted samples and/or anion-inhibited membranes (hereafter referred to as treated membranes) were obtained as described elsewhere [11,22,27]. Samples, containing 250 μM Chl, were incubated in the dark for 60 min in CO_2-free treatment buffer under a stream of N_2 (80%) and O_2 (20%) that had passed through a column of soda-lime and ascarite to facilitate the removal of any trace of CO_2, and through a water column to prevent evaporation of the sample. The treatment buffer contained CO_2-free 300 mM sorbitol, 25 mM sodium formate, 10 mM NaCl, 5 mM $MgCl_2$ and 10 mM sodium phosphate (pH 6.0). However, the reaction medium contained CO_2-free 100 mM sorbitol, 10 mM sodium formate, 10 mM NaCl, 5 mM $MgCl_2$, 20 mM buffer (Mes, pH 6.5; Hepes, pH 7.5–7.6), 100 μM methyl viologen and 0.1 μM gramicidin. The treated samples were maintained under a CO_2-free gas flow throughout the course of the measurement.

Restored membranes were obtained by adding 5 mM HCO_3^- to a 2 ml aliquot of the treated stock. After a 2 min dark incubation these membranes were transferred to the reaction medium which also contained 5 mM HCO_3^- and which had been brought to the correct pH just prior to the measurement. Control membranes were obtained by omitting formate from the treatment and reaction media and not CO_2-depleting these buffers. In the case of the control the incubation pH was also raised to pH 7.5.

All measurements were made on a sample diluted to contain 5 μM Chl in a final volume of 100 ml in a dark stirred vat. A flow cuvette was filled from the vat by computer control.

The kinetics of the decay of variable Chl *a* fluorescence were measured at 685 nm (10 nm bandwidth) by a weak measuring flash which could be fired at variable times after each of a series of actinic flashes. The measuring flash (Stroboslave 1593A, General Radio) sampled approx. 1% of the PS II centers present [28]. Both the actinic flash (FX-124, EG and G) and the measuring flash were blocked by Corning CS4-96 filters, and were of 2.5 μs duration at half-maximal peak height. Details of the instrument are described by Eaton-Rye [29]. An identical instrument and the experimental technique has been described earlier [30].

The relationship between variable Chl *a* fluorescence and $[Q_A^-]$ is non-linear [31]. The half-times ($t_{1/2}$) for Q_A^- oxidation were determined after calculating the concentration of Q_A^- from Eqn. 1 [32]:

$$\frac{F - F_0}{F_m - F_0} = \frac{(1-p)q}{1-pq} \quad (1)$$

where F is the fluorescence yield at time t; F_0 is the fluorescence yield when all Q_A is in the oxidized state; F_m is the maximum fluorescence yield when all Q_A is the reduced state; p, the connection parameter, is taken as the probability of the intersystem energy transfer; and q is the fraction of the closed reaction centers (i.e., $q = 1$ when Q_A^- is maximum). The value of p was taken as 0.5 [33]. Our analysis does not include further refinement discussed by Paillotin [34].

The time at which $[Q_A^-]$ is 50% of maximum $[Q_A^-]$ (at $t = 0$), after a flash, is labeled as t_{50}. All half-times, $t_{1/2}$, given together with their amplitudes, are obtained from plots of log $[Q_A^-]$ as a function of time after evaluation of fast and slow components.

Results and Discussion

The effect of pH on Q_A^- oxidation after flash 1 and flash 2

Chlorophyll *a* fluorescence decays, monitoring the oxidation of Q_A^-, following a single actinic flash, are presented in Fig. 1. The data for two representative experiments (a, b and c, d) at pH 6.5 and at pH 7.5 are shown. Reversibility and full restoration of the Q_A^- oxidation rate by HCO_3^- addition to treated membranes are shown by the almost identical curves for the control and restored samples. Qualitatively similar results were obtained following flash 2 (data not shown).

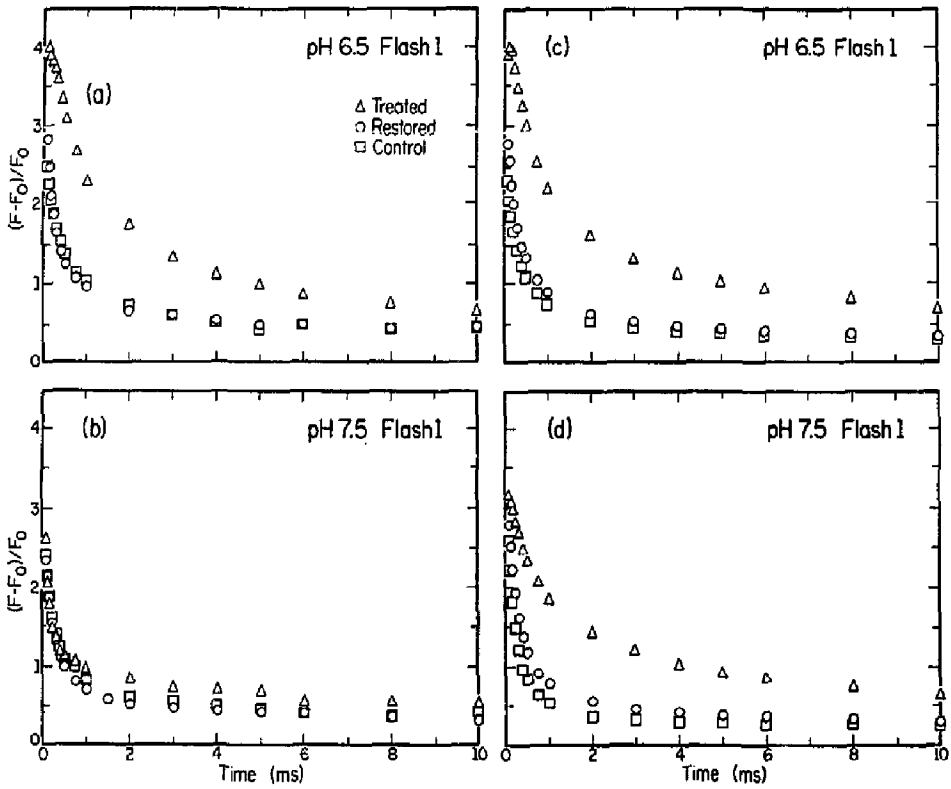

Fig. 1. Decay of variable chlorophyll *a* fluorescence after a single actinic flash at pH 6.5 and pH 7.5. F_0 is the Chl *a* fluorescence yield from the measuring flash with all Q_A^- oxidized and F is the yield at the indicated time after the actinic flash. Two experiments from different batches of spinach are shown with measurements at pH 6.5 (a, c) and pH 7.5 (b, d). The calculated times (t_{50}) at which $[Q_A^-]$ is 50% of maximum $[Q_A^-]$, after a flash, are: (a) for treated membranes, 2.2 ms; for restored membranes, 520 μs; for control membranes, 550 μs; (b) treated, 360 μs; restored, 340 μs; control, 320 μs; (c) treated, 2.4 ms; restored, 390 μs; control, 280 μs and (d) treated, 1.7 ms; restored, 350 μs; control, 230 μs.

At pH 6.5 the two experiments in Fig. 1 yield practically identical results. The times (t_{50}) at which $[Q_A^-]$ is 50% of the maximum $[Q_A^-]$ after a flash in the treated membranes are 2.2 ms in Fig. 1(a) and 2.4 ms in Fig. 1(c), whereas in the control the t_{50} is approx. 550 μs. At pH 7.5, the t_{50} in control membranes is approx. 320 μs, whereas a difference is observed in the treated case in the two experiments: the t_{50} is 360 μs (from Fig. 1 (b)) and 1.7 ms (from Fig. 1 (d)). However, in both cases a faster rate of Q_A^- oxidation is seen over that observed for the pH 6.5 examples. The differences, observed in Figs. 1 (b) and 1 (d), were accompanied by differences in the oxygen evolution rates of PS II particles from the same spinach: 400 μmol O_2 per mg Chl per h vs. 250 μmol O_2 per mg Chl per h (see Fig. 2 in Chapter II of Ref. 35). This emphasizes the maximum possible variability in the samples used in this study.

The maximum variable Chl *a* fluorescence, $(F_{max} - F_0)/F_0$ for Fig. 1 was 4.5. However, apparent differences are seen in the intersection of the ordinate by the fluorescence decay curves, particularly in the treated cases. These arise from (a) the limitation in collecting data until 70 μs after the xenon flash has been fired (the tail of the flash lamp interferes with the measurements); and (b) fluorescence quenching by P-680$^+$ formed due

to equilibration between Z^+, the oxidized form of the electron donor Z, and P-680, the reaction center chlorophyll (see, e.g., Refs. 36–38).

An increase in the time (t_{50}) at which $[Q_A^-]$ reaches a value of 50% of the maximum $[Q_A^-]$ in treated membranes, after one or two actinic

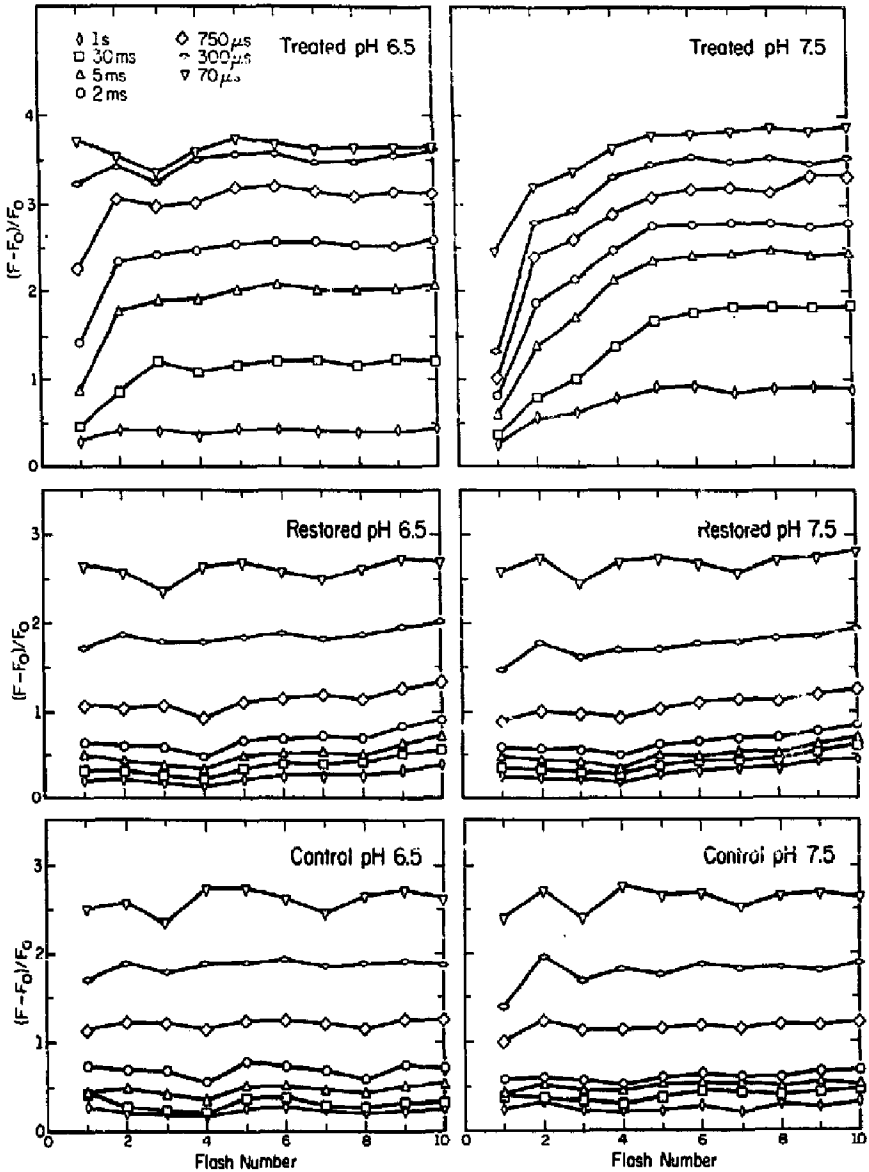

Fig. 2. Variable chlorophyll *a* fluorescence as a function of flash number. The figure shows the HCO_3^- reversible effect in treated membranes at pH 6.5 and pH 7.5. The flash frequency was 1 Hz. The times indicated are when the measuring flash was fired.

flashes, suggests that the apparent forward rate constants for electron flow to either Q_B or Q_B^- must decrease. This is most probably due to a large conformational change in the quinone acceptor complex (e.g., Refs. 11 and 19–21). Our results suggest that such a conformational change would be pH dependent as the inhibited rate of Q_A^- oxidation, after both flashes, is faster at pH 7.5 than at pH 6.5 (Fig. 1; also see Figs. 2 and 3).

One possible consequence of such a conformational change could be an inhibition of the protonation steps associated with plastoquinol formation. After $Q_A^-Q_B \rightleftharpoons Q_AQ_B^-$ electron transport occurs, Q_B^- is protonated: $Q_AQ_B^- + H^+ \rightleftharpoons Q_AQ_B(H^+)$. The equilibrium constant for this reaction has been measured and found to range from 95 at acidic pH to 3.5 at basic pH [39]. In contrast, this equilibrium in treated membranes has been shown to exhibit a two-fold shift to the left at pH 6.0 [40]. This would be consistent with a decrease in the extent of protonation at the Q_B site in treated membranes. It is, therefore, possible that HCO_3^- participates directly in the mechanism of Q_B^- protonation. Alternatively, replacement of HCO_3^- by formate (HCO_2^-) or nitrite (NO_2^-) may produce a conformational change which inhibits this protonation step whether or not HCO_3^- itself is a proton donor. Our data do not allow us to discriminate between these two possibilities.

The second protonation step accompanying plastoquinol formation may also be inhibited. This is also possible whether or not HCO_3^- itself is a proton donor and such an effect would be expected to lead to an inhibition of plastoquinol exchange with the plastoquinone pool. This has been suggested earlier [6,22].

The effect of pH and flash number on chlorophyll a fluorescence yield

The identical results for the variable Chl *a* fluorescence yield ($F - F_0/F_0$) as a function of flash number for restored and control samples (Fig. 2) show the reversibility and full restoration of flash dependence by HCO_3^- addition to treated membranes at both pH 6.5 and pH 7.5. The samples for these experiments were the same as used for data in Fig. 1 (a) and 1 (b). The oscillation observed in the control and restored data arise from at least two causes. A binary oscillation arises from the differential rates of Q_A^- oxidation by either Q_B after an odd number of flashes or Q_B^- after an even number [30]. Superimposed upon this binary oscillation, and seen at 70 μs after the flash, is a period-of-4 oscillation arising from the cycling of the S-states associated with the water-oxidation process [41]. The different S-states re-reduce Z^+ (Z being the electron donor to P-680$^+$) at different rates and different equilibria are involved [42]. In turn, this affects the rereduction of P-680$^+$ by Z after an actinic flash and the associated equilibrium between these two species (see e.g., Ref. 38). The resulting changes in the P-680$^+$ population then impose the period-of-4 oscillation. These oscillations are altered in the treated membrane at pH 6.5, and are completely lost at pH 7.5.

In summary, at both pH 6.5 and pH 7.5 the two-electron gate turnover proceeds without obvious impediment in the restored and control membranes. However, the flash pattern for the treated membranes at these pH values indicates that successive turnovers of the two-electron gate in these centers proceed more slowly at the alkaline pH than at the acidic pH (Fig. 2). In the treated membranes at pH 6.5, the fluorescence yield from 750 μs to 30 ms after the actinic flash reaches its maximum after the 2nd or the 3rd flash in contrast to the 5th flash at pH 7.5. However, the maximum fluorescence yield (2 ms–1 s) after five flashes is always higher at pH 7.5 than at pH 6.5. Therefore, the oxidation of Q_A^- in treated samples, after two turnovers of the reaction center, is slowed further at pH 7.5 than at 6.5.

A major difference caused by bicarbonate depletion, observed clearly at pH 7.5, lies in the increase in the ratio of $(F - F_0)/F_0$ at 70 μs after the third to that after the second flash (Fig. 2). This can be easily understood if we postulate that the exchange of Q_B^{2-} with Q_B is slowed down and that this effect is related to the role of HCO_3^- in protonation at this site.

The flash number dependence of the time (t_{50}) at which $[Q_A^-]$ is 50% of the maximum $[Q_A^-]$ after a flash in treated membranes from two different batches of spinach are shown in Fig. 3. In each case, the data are best interpreted by a slower Q_A^- oxidation rate at pH 7.5 after two turnovers (flash five and subsequent flashes) of the two-electron

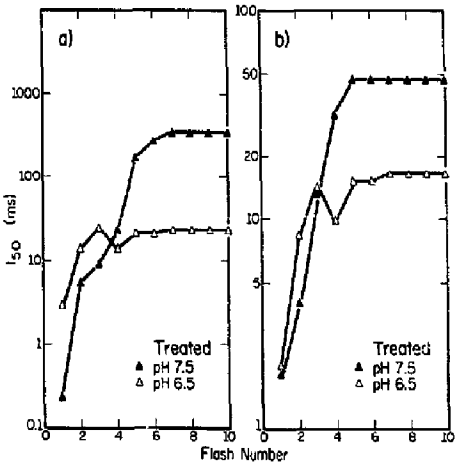

Fig. 3. Plot of the times (t_{50}) at which [Q_A^-] is 50% of maximum [Q_A^-] in treated membranes with measurements at pH 6.5 and pH 7.5. Two experiments from different batches of spinach are shown. The flash frequency was 1 Hz.

gate than that at pH 6.5. At pH 6.5, although the t_{50} for Q_A^- oxidation is not slowed further after one turnover of the two-electron gate (three flashes), it is still faster than that at pH 7.5. The 'transition', observed after three flashes, is in agreement with earlier observations [8,11,16]. Qualitatively, the same results are obtained with the two batches of spinach (one used for Figs. 3(a) and 2; and the other for Figs. 3(b) and 4). The differences between the two batches lie only in the actual values of t_{50} (see below). In addition, the rate of Q_A^- oxidation after the first two flashes is faster at pH 7.5 than at 6.5, but it is reversed at higher flash numbers. These results suggest that two counterbalancing phenomena exist. Firstly, a pH-dependent conformational change possibly exists, resulting in a faster oxidation of Q_A^- at pH 7.5 than at pH 6.5; and secondly, the removal of HCO_3^- may inhibit the protonation reactions associated with plastoquinol formation. At 1 Hz, the inhibition of the protonation reaction is more marked at pH 7.5 than at pH 6.5 (Figs. 2 and 3).

The kinetics of Q_A^- oxidation in treated membranes have been analyzed for discrete exponential components [16,43]. Caution, however, must be exercised when interpreting such data, as it is uncertain whether the processes reflected in the resulting components are first order or not. This approach to the data yields an apparent biphasic decay with a component in the 300–1000 μs range and a second component in the 5–20 ms range with additional components in the 0.1–10 s range. The variation in the time (t_{50}) at which [Q_A^-] is 50% of the maximum [Q_A^-], after a flash, shown for the treated membranes in Fig. 3(a) and 3(b), appears in large part to be correlated with the relative contribution of these additional slow components. In Fig. 3(a) t_{50} for Q_A^- oxidation at pH 7.5 after the fifth actinic flash is 135 ms and in Fig. 3(b) the corresponding t_{50} is 47 ms.

The effect of pH and flash frequency on chlorophyll a fluorescence yield

At 1 Hz, at 30 ms after the actinic flash in a train of flashes (Figs. 2 and Fig. 4(a) and (d)), the fluorescence yield, proportional to [Q_A^-], reaches its maximum after flash 5 at pH 7.5 and after flash 3 at pH 6.5, as noted earlier. However, Fig. 4(a) demonstrates that even at pH 6.5 the number of turnovers necessary before maximal [Q_A^-] is reached is extended to flash 4 or 5 if the flash frequency is increased to 5 Hz. It is therefore possible that a dark-time of 200 ms (at 5 Hz) is insufficient, in the protonation hypothesis, for an equal amount of protonation to occur at the electron acceptor side of PS II than when 1 s (1 Hz) is allowed to elapse between flashes.

The Q_A^- oxidation data for the treated, control and restored samples after flash 5 at pH 6.5 and at 5 Hz, as a function of time (log scale) show the full reversibility of the HCO_3^- treatment (Fig. 5). The time (t_{50}) at which [Q_A^-] is 50% of maximum [Q_A^-], after a flash, for the restored and control decays was approx. 600 μs which was quite similar to that measured for flash 1 in Fig. 1(a) and (c) and suggests that no obvious inhibition is caused at this frequency in these membranes. The main result is that in treated membranes the t_{50} at 1 Hz is 22 ms, but at 5 Hz, it is increased to approx. 170–180 ms.

Dependence of the t_{50} of Q_A^- decay on the flash frequency at pH 6.5 revealed that in treated membranes it increased continuously from 10 ms at 0.5 Hz to 175 ms at 4 Hz, whereas at pH 7.6 it was already high (42 ms) at 0.5 Hz and reached its maximum (250 ms) even at 1 Hz. Similar results

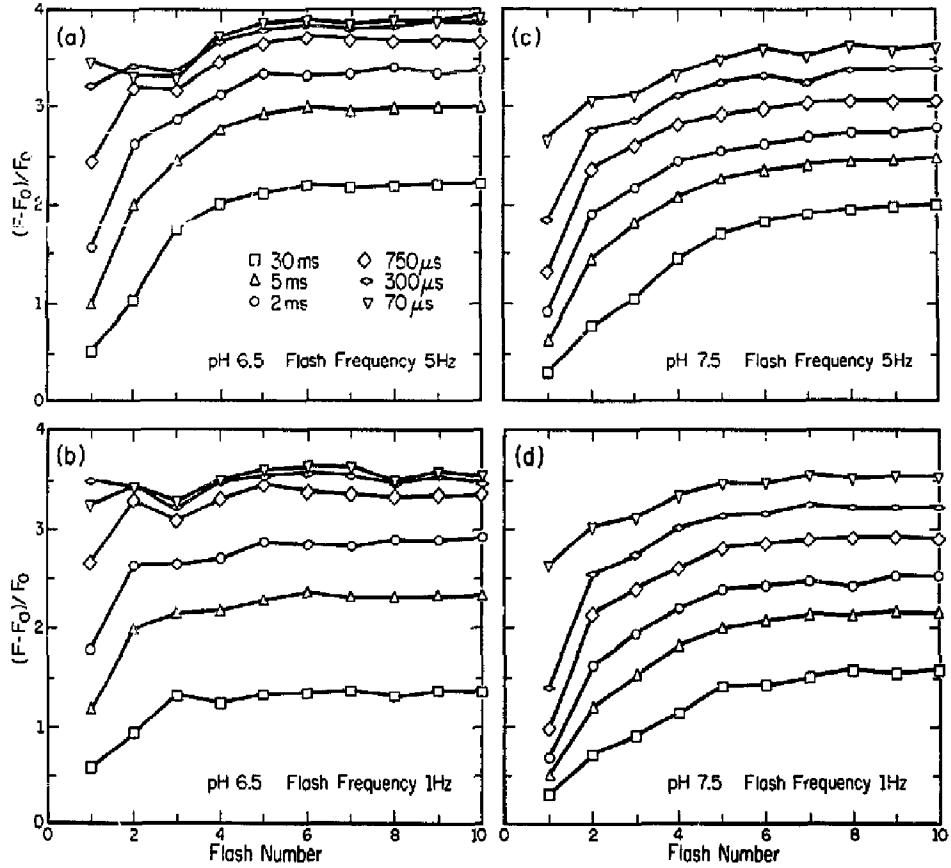

Fig. 4. Variable chlorophyll *a* fluorescence yield as a function of flash number at two frequencies (1 or 5 Hz) and at pH 6.5 and pH 7.5.

were obtained with another batch of spinach where the t_{50} at 1 Hz was 18 ms (at pH 6.5) and 180 ms (at pH 7.6). The almost 10-fold increase in the t_{50} value was due to an increase in the 0.1–10 s decay components. No dependence on flash frequency was observed for the decay of Q_A^- reoxidation in the control as well as in the restored samples: the range of t_{50} values for Q_A^- decay was 480–660 μs.

Conclusions

Major observations

The results presented in this paper establish four major points with respect to the HCO_3^--reversible inhibition seen in treated membranes: (1) the oxidation of Q_A^- or Q_B or Q_B^-, following one or two actinic flashes respectively, exhibits a smaller value for the time (t_{50}) at which $[Q_A^-]$ is 50% of maximum $[Q_A^-]$ at pH 7.5 than at pH 6.5 (Figs. 1, 2 and 3); (2) the characteristic oscillations observed in the fluorescence flash pattern, generated by assaying the Chl *a* variable fluorescence $(F - F_0)/F_0$ at specific times after an actinic flash and plotting these data as a function of flash number, are lost (Figs. 2 and 4); (3) the slowest oxidation of Q_A^-, as measured by the decay of Chl *a* fluorescence, depends on both pH and flash number (Figs. 2–4); at pH 6.5, the system is

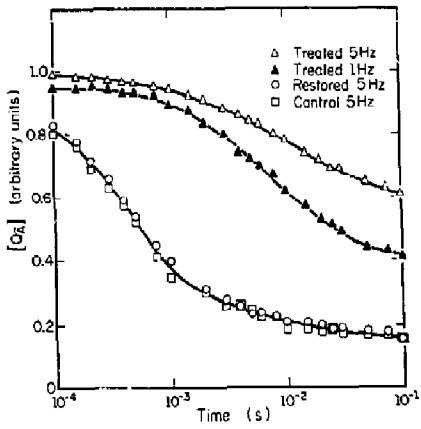

Fig. 5. Oxidation of Q_A^- after the fifth actinic flash in a train of five flashes given at 5 Hz (open symbols) or 1 Hz (closed triangles). The data are expressed as $[Q_A^-]$ against time plotted on a logarithmic scale. A value of 1.0 on the ordinate is equivalent to all centers being in the state Q_A^-. The pH was 6.5.

blocked after one turnover of the two-electron gate, whereas at pH 7.5, the oxidation of Q_A^- is slowed to an even greater degree after two turnovers of the two-electron gate; and (4) the decay of Chl *a* fluorescence also depends on the actinic flash frequency (Figs. 4 and 5); at 5 Hz, the bottle-neck reaction associated with Q_A^- oxidation at pH 6.5 is slowed to the same extent, after two turnovers of the two electron gate, as that observed at pH 7.5.

Inactive centers

Jursinic and Stemler [43] were the first to report the existence of a significant fraction of PS II centers remaining closed to photochemistry after a single actinic flash in treated membranes. In our samples, approx. 20% of the corrected variable Chl *a* fluorescence, proportional to $[Q_A^-]$, decays in the 0.1–10 s range. In the control and restored samples a value of about 10–15% is typical and appears to be fairly independent of flash number. This fraction of inactive centers is probably related to inactive centers associated with PS II heterogeneity (see, e.g., Ref. 44). However, at pH 6.5 for flash 5, from a train of actinic flashes given at 1 Hz, the 20% amplitude is increased to 55% in the treated membranes and the corresponding value at pH 7.6 is 70%. At an actinic flash frequency of the 5 Hz the amplitude of the slow components (0.1–10 s) is approx. 72% at both pH 6.5 and pH 7.6 in treated membranes. We speculate, therefore, that the inactivity of the 'inactive' PS II centers may be due to the absence of bound HCO_3^-. For a discussion of the various fluorescence decay components, see Van Gorkom [45].

Protonation of the secondary plastoquinone acceptor and a working hypothesis

The protonation of Q_B^- in thylakoid membranes is suggested to involve a dissociable group on the D1 and D2 reaction center proteins (for a discussion of D1 and D2, see Ref. 46) contributing to the binding site for Q_B, rather than protonation of Q_B^- itself [47]. Such a mechanism was first proposed by Wraight [48] in bacterial reaction centers. Robinson and Crofts [39] have measured a pK of 6.4 for the dissociable group in thylakoid membranes when Q_B is oxidized; this pK is shifted to 7.9 upon the formation of Q_B^-. The protonation of Q_B^- is suggested to become the rate-determining step in plastoquinone reduction to plastoquinol above pH 7.9 [47].

We suggest that HCO_3^- participates in this protonation step. Replacement of HCO_3^- by formate appears to induce a large conformational change in the quinone acceptor complex (see, e.g., Ref. 18). From our data in Fig. 1 it would appear that the conformational change may be pH dependent, and it is possible that at pH 6.5 the Q_B binding domain is so altered that Q_B^- is no longer readily protonated. This could occur if the dissociable protein group, postulated to protonate Q_B^-, decreased its dissociation constant. At pH 7.5, however, the dissociable group is better able to protonate Q_B^- and, taken together with the observation that the conformational change is less inhibitory at alkaline pH, the Chl *a* fluorescence decay, after a single actinic flash, more closely resembles that observed in control membranes.

Blubaugh and Govindjee [7] have suggested that the hydroxyl group in HCO_3^- may function in protonation. It is possible that HCO_3^- may donate a proton to the putative dissociable protein group. In treated membranes, however, HCO_3^- has been replaced by formate and thus no proton is available. As mentioned earlier, HCO_3^- may also participate in the second protonation step

associated with plastoquinone formation. Thus, in successive turnovers of the reaction center subsequent protonation of Q_B^- and possiby Q_B^{2-} would be dependent on the bulk pH. Additionally at high actinic flash frequencies (e.g., 5 Hz) the extent of protonation would be expected to be less, particularly at alkaline pH, than at low frequencies (e.g., 1 Hz). Thus, this hypothesis explains qualitatively the pH-dependent, flash- and frequency-dependent behavior of HCO_3^- reversible changes observed in this work. Furthermore, the frequency dependence observed for treated membranes may explain the 7–10-fold HCO_3^- reversible inhibition observed, on steady-state oxygen evolution measurements, in identical preparations to those employed in this study [22,29]. The excitation frequency was on the order of 200 Hz in the oxygen measurements. Therefore the fraction of centers turning over rapidly (i.e., smaller than 5 ms) in treated membranes may be reduced even further than observed here at an excitation frequency of 5 Hz.

The results presented here do not rule out additional explanations for our observations. In particular the behavior of Q_A^- oxidation after a single actinic flash may result from a pH dependent conformational change which increases the distance between Q_A and Q_B and thus slows the rate of electron transfer. The contribution from the association constant for Q_B binding to the quinone acceptor complex is unknown in treated membranes. Neither can we eliminate the possibility that HCO_3^- may also affect the rate of exchange between plastoquinone and plastoquinol at the Q_B binding site. Both of these latter points would be contributing factors to the observed increase in t_{50} after one or two turnovers of the two-electron gate.

While the site of action (electron transfer through the plastoquinone acceptors of PS II (Refs. 8, 16 and 37; see also this paper), and the active species (HCO_3^-, and not CO_2 nor CO_3^{2-} [7]) of the bicarbonate effect are known, further experimentation is still required to understand the mechanism of this phenomenon.

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