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THE REDUCTION OF THE OXYGEN-EVOLVING SYSTEM IN CHLOROPLASTS BY THYLAKOID COMPONENTS

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Using thoroughly dark-adapted thylakoids and an unmodulated Joliot-type oxygen electrode, the following results were obtained. (i) At high flash frequency (4 Hz), the oxygen yield at the fourth flash (Y_4) is lower compared to Y_3 than at lower flash frequency. At 4 Hz, the calculated S_0 concentration after thorough dark adaptation is found to approach zero, whereas at 0.5 Hz the apparent $S_0/(S_0 + S_1)$ ratio increases to about 0.2. This is explained by a relatively fast donation ($t_{1/2} = 1.0–1.5$ s) of one electron by an electron donor to S_2 and S_3 in 15–25% of the Photosystem II reaction chains. The one-electron donor to S_2 and S_3 appears to be rereduced very slowly, and may be identical to the component that, after oxidation, gives rise to ESR signal II_s. (ii) The probability for the fast one-electron donation to S_2 and S_3 has nearly been the same in triazine-resistant and triazine-susceptible thylakoids. However, most of the slow phase of the S_2 decay becomes 10-fold faster ($t_{1/2} = 5–6$ s) in the triazine-resistant ones. In a small part of the Photosystem II reaction chains, the S_2 decay was extremely slow. The S_3 decay in the triazine-resistant thylakoids was not significantly different from that in triazine-susceptible thylakoids. This supports the hypothesis that S_2 is reduced mainly by Q_A^-, whereas S_3 is not. (iii) In the absence of CO_2/HCO_3^- and in the presence of formate, the fast one-electron donation to S_2 and S_3 does not occur. Addition of HCO_3^- restores the fast decay of part of S_2 and S_3 to almost the same extent as in control thylakoids. The slow phase of S_2 and S_3 decay is not influenced significantly by CO_2/HCO_3^-. The chlorophyll *a* fluorescence decay kinetics in the presence of DCMU, however, monitoring the Q_A^- oxidation without interference of Q_B, were 2.3-fold slower in the absence of CO_2/HCO_3^- than in its presence. (iv) An almost 3-fold decrease in decay rate of S_2 is observed upon lowering the pH from 7.6 to 6.0. The kinetics of chlorophyll *a* fluorescence decay in the presence of DCMU are slightly accelerated by a pH change from 7.6 to 6.0. This indicates that the equilibrium Q_A^- concentration after one flash is decreased (by about a factor of 4) upon changing the pH from 7.6 to 6.0. When direct or indirect protonation of Q_B^- is responsible for this shift of equilibrium Q_A^- concentration, these data would suggest that the pK_a value for Q_B^- protonation is somewhat higher than 7.6, assuming that the protonated form of Q_B^- cannot reduce Q_A.

Introduction

The oxygen yield pattern caused by excitation of dark-adapted thylakoids with a single-turnover flash train reveals a characteristic oscillation with a periodicity of four, having a maximum at the

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Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea, PS II, Photosystem II; DCPIP, 2,6-dichlorophenolindophenol; Mes, 4-morpholineethanesulphonic acid.

third flash [1,2]. This can be explained by a four-step oxidation of water to O_2 with the assumptions that the oxygen evolving system contains one oxidizing redox equivalent (referred to as state S_1) after dark adaptation and that the flash-induced advancement of the 'redox clock' involves a certain probability of misses and double turnovers (α and β, respectively). Oxygen can be evolved only after the redox state S_4 is attained. The redox states S_2 and S_3 are unstable in the dark and decay, probably via univalent reduction steps, to S_1. S_0, is, however, assumed to be rather stable and not to be oxidized easily to the S_1 state [3]. A relatively high oxygen yield in the fourth flash has often been attributed to Photosystem II (PS II) reaction chains that were in S_0 state after dark adaptation. However, it is shown here that in dark-adapted thylakoids the apparent S_0/S_1 ratio is greatly affected by a fast S_2- and S_3-decay ($t_{1/2} = 1.0$–1.5 s) occurring only once in approx. 20% of the PS II chains. After correction for this fast S_2/S_3 decay, the S_0/S_1 ratio after thorough dark adaptation approaches zero.

This is in line with conclusions from Velthuys and Visser [4], who showed that the change in flash pattern observed after addition of DCPIP and ascorbate is not due to a reduction of S_1 into S_0 in the dark, but to the reduction of a component, which could reduce part of S_2 and S_3 in the dark time between the flashes. The oxidation of the S_2/S_3-reducing component gives rise to EPR signal II_s [12,4]. Velthuys and Visser also suggested that all centres revert to the state S_1 in the dark, and that the apparent S_0 fraction present after dark adaptation might be due to S_2 and S_3 reduction by the component that gives rise to EPR signal II_s [4].

The S_3 decay was found to be rather independent of the redox state of the plastoquinone pool, whereas the S_2 decay rate was found to increase upon reduction of plastoquinone, suggesting reduced quinones as the electron source for S_2 reduction [5]. Moreover, it has been shown recently by elegant fluorescence measurements that the reduced primary quinone Q_A^- is the donor for S_2 reduction [6]. Accordingly, the S_2 decay should become modified by a change in the redox equilibrium $Q_A^- \cdot Q_B \leftrightharpoons Q_A \cdot Q_B^-$, whereas the S_3 decay is expected to remain unaffected. The data presented in this study confirm this for different thylakoids (triazine-resistant, CO_2-depleted).

Materials and Methods

Thylakoid isolation

Leaves from 2.5–3 weeks old pea plants (*Pisum sativum* L.) grown in a growth chamber were ground in a Sorvall homogenizer for 2–3 s, using a buffer (the isolation/reaction buffer) that consisted of 50 mM Tricine-NaOH/10 mM NaCl/5 mM $MgCl_2$/0.3 M sorbitol. The buffer was at pH 7.6. The homogenate was filtered through four layers of fine-meshed nylon cloth and centrifuged for 5 min at $2000 \times g$. The pellet was resuspended in a medium consisting of 10 mM Tricine-NaOH/10 mM NaCl/5 mM $MgCl_2$ (end pH 7.6) bringing about an osmotic shock.

After centrifugation (5 min, $2000 \times g$) the stroma-free thylakoids were resuspended in the isolation/reaction buffer. For the experiments with triazine-resistant and -susceptible biotypes, thylakoids were isolated from 3–6-week-old triazine-resistant and -susceptible *Brassica napus* leaves analogously. Chlorophyll concentration determinations at 645 and 663 nm in 80% acetone were carried out as described by Arnon [7].

CO_2 depletion

A pea thylakoid suspension in isolation/reaction buffer was diluted into a CO_2-free medium at pH 6.0 consisting of 50 mM Mes-NaOH/25 mM HCOONa/10 mM NaCl/5 mM $MgCl_2$/0.2 M sorbitol, and allowed to incubate for 1 h in the dark at room temperature. After this treatment, the ferricyanide Hill reaction at saturating light intensity was very low (less than 20 μmol O_2/mg Chl per h) and was increased by a factor of 10 or more by addition of 5 mM $NaHCO_3$ (see Ref. 8 for a review on this phenomenon). The oxygen yield measurements with CO_2-less thylakoids were performed in the Mes medium at pH 6.0.

Oxygen electrode

An unmodulated Joliot-type O_2 electrode was used as described in Ref. 9. The thylakoid suspension (0.8 mg chlorophyll/ml) on the Pt-electrode was separated from the upper buffer compartment in contact with the Ag|AgCl electrode by a single

dialysis membrane. Thylakoids were transferred from the stock suspension, kept in absolute darkness, to the electrode surface in very dim light. No significant amount of turnovers of the oxygen evolving system appeared to occur upon transfer. After 5 min of additional dark adaptation, the thylakoids were illuminated by saturating Xe flashes (12 μs half-width) using a Stroboslave 1539-A (General Radio). The signals were amplified and fed into a Nicolet Explorer III storage oscilloscope (1–5 ms per point). The signals were written out, using an HP 7015 B X–Y recorder, and the amplitudes of the signals were measured. For more details, see Ref. 10.

Calculation of α and β and the distribution of S states after dark adaptation was done by fitting the experimental Y_1 to Y_{10} data to flash yields calculated from a set of α, β and $S_0/(S_0 + S_1)$ by least-square fit with a HP 9825B computer, using the classical Kok model [2].

For the S_2, S_3 and S_0 decay measurements, the thylakoids were preilluminated with one, two or three flashes (4 Hz), respectively, followed by a flash train of ten flashes (4 Hz) after a certain time interval. The relative amount of S_2 was calculated from the oxygen yield in the second flash of the flash train (Y_2), corrected for the Y_2 obtained without preflashes ($Y_{2,c}$), divided by the average oxygen evolution in the 2nd–5th flash ($\overline{Y}_{2-5}$). The relative amount of S_3 was obtained from Y_1 divided by $\overline{Y}_{1-4}$. Analogously, the relative amount of S_0 was estimated from Y_4 divided by $\overline{Y}_{1-4}$. Fitting the flash patterns to populations of S_0–S_3 after preflashes just before the flash train (using the control values of α and β) with the help of a computer, yielded the same decay rates of S_2 and S_3 as those calculated as described above.

Results and Discussion

It was observed that the flash pattern of oxygen evolution depended on the flash frequency. At 0.5 Hz flashes, Y_4 was significantly higher and Y_3 lower than at 4 Hz flashes (Fig. 1). Flash patterns at 1 and 2 Hz are intermediate to the two shown in Fig. 1. Numerical fitting of the experimental data obtained at the two flash frequencies yielded $S_0/(S_0 + S_1) = 0.03$, $\alpha = 0.13$ and $\beta = 0.04$ for the 4 Hz pattern, and $S_0/(S_0 + S_1) = 0.23$, $\alpha = 0.13$ and $\beta = 0.04$ for the 0.5 Hz pattern. Therefore, a dependence of the apparent S_0 fraction after dark adaptation on the flash frequency is obtained. However, it is not comprehensible that the real S_0 fraction after dark adaptation would depend on the flash frequency. One possibility to explain this seemingly anomalous result is the assumption of the donation of one electron by an unknown donor to the oxygen-evolving complex in certain reaction chains at such a rate that the donation is observed at 0.5 Hz flashes, but not (or much less) at 4 Hz flashes.

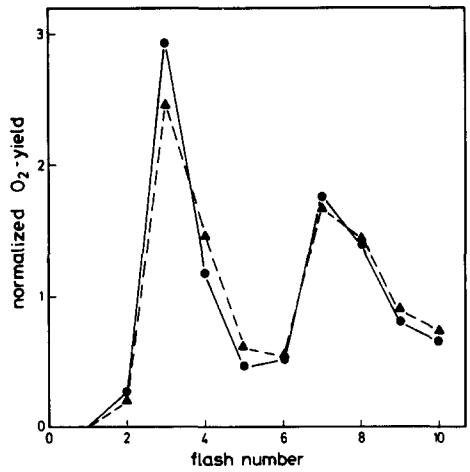

Fig. 1. Oxygen production as a function of flash number by thoroughly dark-adapted pea thylakoids in isolation/reaction medium. The flash frequency was 4 (●) or 0.5 (▲) Hz. The data are normalized to an average O_2 evolution of 1.0 in the first ten flashes.

Therefore, the decay of S_2 and S_3 was measured. The results are shown in Fig. 2. The decay is clearly biphasic; the fast phase (with an amplitude of 15–25% of the total, depending on the thylakoid preparation) generally shows a half-time of 1.3–1.6 s, whereas the $t_{1/2}$ of the slow phase is 28–35 s for S_2 and 80–110 s for S_3. A similar biphasic pattern of S_3 decay in thylakoids has been published in Ref. 11. Sometimes, a very slow component in S_2 or S_3 decay was observed (less than 15% of the total amplitude and a $t_{1/2}$ in the order of a few minutes) (data not shown).

The results reported above indicate that the apparent $S_0/(S_0 + S_1)$ ratio calculated from data obtained at low flash frequencies does not reflect

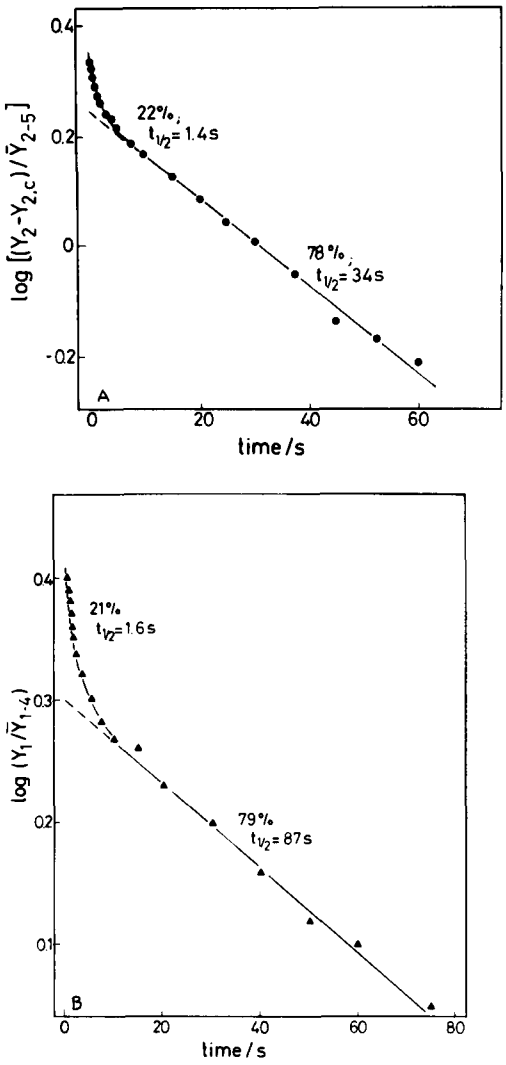

Fig. 2. Decay kinetics of the S_2 (A) and S_3 (B) states in pea thylakoids in isolation/reaction medium. In order to provide the reader with a better view on the fast phase of the decay, the time-scale has been shortened.

the real S_0/S_1 state distribution after dark adaptation, but is increased due to a fast decay of part of S_2 and S_3. The fast electron donation of an electron to the S_2 or S_3 states in an oxygen-evolving complex appears to occur only once after dark adaptation; otherwise the α parameter should increase initially upon decreasing the flash frequency to 0.5 Hz, and the reaction chains with endogenous donor to S_2 or S_3 would disappear gradually from the flash pattern when decreasing the flash frequency from 0.75 Hz to lower values, leading to a decreasing α. This is not observed (data not shown). Therefore, the fast reduction can occur only once (either at S_2 or at S_3) in a reaction chain.

The nature of the one-electron carrier, which is not rereduced rapidly and which probably is in the vicinity of the O_2-evolving complex and serves as a fast electron donor to S_2 and S_3, is still unknown. However, the plastoquinol-like thylakoid component which reduces S_2 and S_3 on the seconds time-scale and which, after oxidation, gives rise to EPR signal II_s (see Ref. 12) is a likely candidate, since it is also involved in S_2- and S_3-reduction in the presence of DCPIP/ascorbate [4]. It should be noted that only approx. 20% of the PS II electron-transport chains appears to be connected to the one-electron donor to S_2 and S_3; this result is not only observed in pea thylakoids, but also in spinach and rape seed thylakoids (data not shown). However, the concentration of the component responsible for signal II_s was suggested to be equal to that of P-700 [13]. It is possible that under the conditions used only 20% of the reaction chains have the component, which gives rise to EPR II_s, in the reduced rather than in the semi-oxidized form.

Since in a 4 Hz flash sequence some of the S_2 and S_3 will decay between the flashes, the real S_0 fraction after dark adaptation will be even less than 0.03 (calculated from the flash pattern in Fig. 1). However, it should be noted that the apparent S_0 fractions have been calculated under the assumption that α is not a function of flash number. In case, for example, α is higher when the $Q_A \cdot Q_B$ complex is in the semireduced form ($(Q_A \cdot Q_B)^-$) than when the $Q_A \cdot Q_B$ complex is fully oxidized (which is probable because the 'free' electron in the $(Q_A \cdot Q_B)^-$ complex has a certain probability of being located on Q_A, resulting in a 'closed' reaction center), then the α at the odd flashes will be lower than at even flashes. In this case, the calculated S_0 fraction will be somewhat lower than the real S_0 fraction after dark adaptation.

Regardless of these details, these results indicate that most of the $S_0/(S_0 + S_1)$ fractions after dark adaptation calculated in previous papers may have been overestimated. The fast phase of S_2 and S_3 decay reported here had not been observed in most earlier studies because these studies did not include any of the relatively short intervals (less

than 2 s) between preflashes and the flash train, or else used a low flash frequency.

The finding that the calculated S_0 fraction after long dark adaptation is very low (0–0.05) indicates that most of the S_0 is oxidized to S_1 and is not as stable as was hypothesized earlier [3]. The S_0 decay was measured by using three preflashes, followed by a flash train after variable dark time. A few representative flash patterns are shown in Fig. 3. Neglecting differences caused by residual S_2 and S_3 concentrations, it is clear that some part of S_0 has been oxidized to S_1 after 5 min, and that a large part of S_0 has been converted into S_1 state after 20 min. (The polarization voltage of the electrode was turned off until 30 s before the flash train; when the polarization voltage was on during the time between preflashes and flash train, the S_0 oxidation was observed to be somewhat slower. This may have been caused by a difference in O_2 concentration in the thylakoid suspension on the Pt-electrode.) Time intervals longer than 20 min between preflashes and flash train did not change the flash pattern significantly. Although the S_0 fraction after 20 min is low, it does not seem to be zero, since there is still a difference compared to the control pattern.

There are two possibilities to explain this difference: (1) a small part of the S_0 does not convert into S_1; and (2) the parameter α is dependent on the redox state of the $Q_A \cdot Q_B$ complex. The control flash pattern starts out with the $Q_A \cdot Q_B$ complex mainly oxidized, whereas the flash pattern after three preflashes starts out mainly with $(Q_A \cdot Q_B)^-$. At this moment, we cannot distinguish between the two possibilities. The results observed may have arisen from a combination of the two effects.

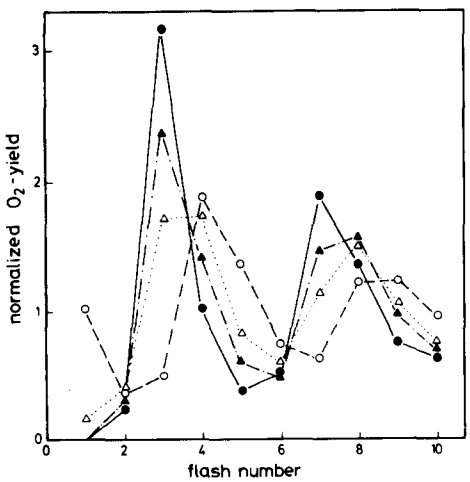

Fig. 3. Flash-induced oxygen evolution pattern of pea thylakoids without preflashes (●), or with three preflashes (frequency 4 Hz) fired immediately before (at 0.25 s) (○), 5 min (△) or 20 min (▲) before the 4 Hz flash train. The polarization voltage of the electrode was turned off until 0.5 min before the flash train. The data are normalized to an average O_2 evolution of 1.0.

The influence of triazine-resistance

It is known that triazine-resistant thylakoids show a rather strongly damped oscillation pattern

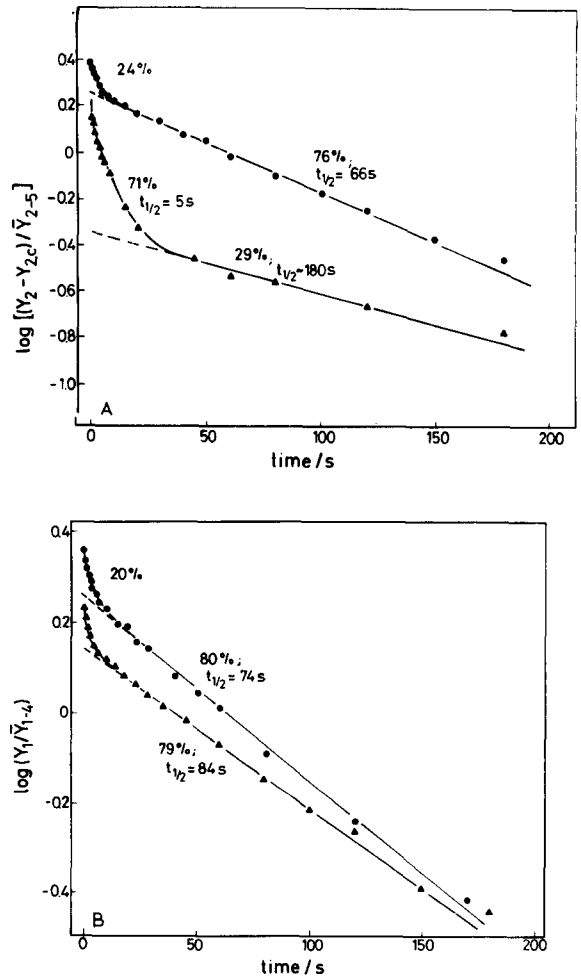

Fig. 4. Decay kinetics of the S_2 (A) and S_3 (B) states in triazine-susceptible (●) and -resistant (▲) thylakoids from *Brassica napus* in isolation/reaction medium.

and a relatively high Y_2 [14]. This was assumed to be due to an increased α and β. However, an increased β is not likely to occur due to the slow reoxidation of Q_A^- [15], unless in resistant thylakoids the flash-induced reduction of Q_2 [16] or X_A [17] is increased. The increased α can be easily understood because of the increased equilibrium Q_A^- concentration [18]. In order to investigate the cause of the high Y_2 and to test the hypothesis of S_2 reduction by Q_A^-, we measured S_2 and S_3 decay kinetics in thylakoids of triazine-susceptible and -resistant biotypes of *Brassica napus*. The results are shown in Fig. 4. The S_3 decay is almost the same in resistant compared to susceptible thylakoids; both show a slow and fast phase. The S_2 decay, however, is different: the susceptible thylakoids behave as pea thylakoids shown in Fig. 2, although the decay kinetics are slower by a factor of 2, whereas the larger part of the S_2 decay is quite fast in resistant thylakoids ($t_{1/2} = 5$–6 s). A small part of S_2, however, decays extremely slowly in resistant thylakoids ($t_{1/2}$ more than 2 min). We cannot distinguish whether the 15–25% fast phase with $t_{1/2} = 1.5$ s is still there, because this effect is largely overshadowed by the phase of 5–6 s.

The ratio of half-times of S_2 decay in triazine-susceptible and -resistant thylakoids ($66/5 = 13$) is likely to represent the factor by which the equilibrium Q_A^- concentration has increased in resistant thylakoids, assuming the S_2 reduction by Q_A^- at maximal Q_A^- concentration to be unchanged. In order to test this and in order to obtain a more absolute value for the Q_A^- equilibrium concentration under various conditions, we measured the decay of the chlorophyll *a* fluorescence in the presence of 5 μM DCMU, and the decay kinetics of 320 nm absorption changes in the presence of DCMU, representing mainly Q_A^- decay. The latter was measured by double-flash experiments [19] using pea thylakoids. The $t_{1/2}$ of the chlorophyll *a* fluorescence in the presence of DCMU was found to be 1.26 ± 0.04 for pea thylakoids, 1.22 ± 0.05 for triazine-susceptible and 1.27 ± 0.03 s for triazine-resistant rape seed thylakoids. Thus, the reaction constant of S_2 reduction by Q_A^- is not changed. The half-time of the 320 nm changes in the presence of DCMU was found to be approx. 2.8 s; this is markedly slower than the half-time of fluorescence decay. This difference is due to the non-linearity between Q_A^- concentration and fluorescence yield. These data indicate that in triazine-susceptible *Brassica napus* thylakoids the equilibrium $[Q_A^- \cdot Q_B]/([Q_A^- \cdot Q_B] + [Q_A \cdot Q_B^-])$ ratio after one flash is $2.8/66 = 0.04$, whereas in resistant thylakoids this ratio is $2.8/5 = 0.56$, which is approx. twice as high as the overall miss parameter of 0.25 that we calculated for the triazine-resistant thylakoids. This implies that in resistant thylakoids the misses are caused mainly by the high equilibrium Q_A^- concentration when the $Q_A \cdot Q_B$ complex is in $(Q_A \cdot Q_B)^-$ state rather than by some effect on the donor side. These data indicate that the flash patterns of triazine-resistant thylakoids have to be fitted by assuming a $Q_A \cdot Q_B$ redox-state-dependent α (low in $Q_A \cdot Q_B$ state, high in $(Q_A \cdot Q_B)^-$ state) rather than by an 'overall' α. The same is probably true for normal thylakoids, although the difference between the two alphas is expected to be much less dramatic.

The very slow component of S_2-decay, on whose cause we do not speculate here, yields a good explanation for the relatively high Y_2 after a few minutes of dark adaptation as observed by Holt et al. [14,20]. We observed that the resistant thylakoids, kept in the dark on ice, showed a decreasing Y_2 in the course of the day. Therefore, a high Y_2 in resistant thylakoids is likely not to be due to an increased double-hit probability [14,20], but to a very stable fraction of S_2.

The effect of CO_2 / HCO_3^-

It is known that absence of CO_2/HCO_3^- in the presence of formate ($HCOO^-$) leads to a reversible inhibition of electron transport at the quinone level [8]. However, the consequences of CO_2/HCO_3^- absence for the equilibrium Q_A^- concentration are still unknown. Under our conditions, the amplitude of the flash pattern at 4 Hz decreased with increasing flash number, probably due to a gradual inhibition of electron transport to PQ, whereas at 0.5 Hz the flash pattern is normal (Fig. 5; compare with Fig. 1). Readdition of 5 mM $NaHCO_3$ restored the normal flash pattern also at 4 Hz frequency (data not shown). The damping of the oscillation is somewhat less than was reported at pH 6.8 [21]. It should be noted that the '$-CO_2$'

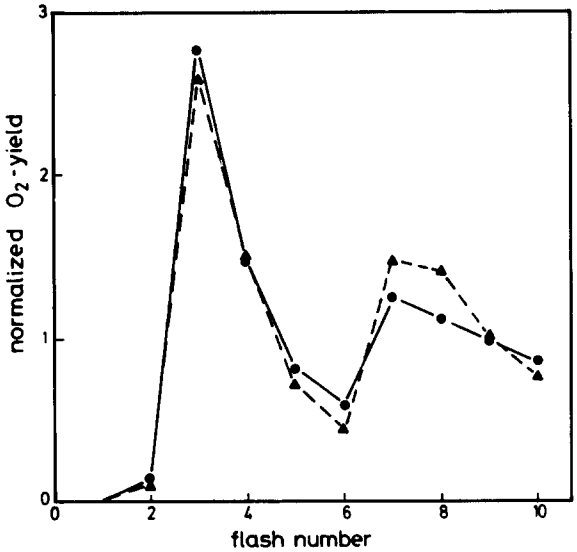

Fig. 5. Oxygen evolution pattern of pea thylakoids in a CO_2-free medium at pH 6.0 containing 50 mM Mes-NaOH/25 mM HCOONa/10 mM NaCl/5 mM $MgCl_2$/0.2 M sorbitol. The flash frequency was 4 (●) or 0.5 (▲) Hz. The data are normalized to an average O_2 evolution of 1.0 in the first ten flashes.

thylakoids used here are not thoroughly CO_2-depleted, because in our arrangement exposure to the air is required to apply thylakoids to the electrode. At saturating light intensity a 10–15-fold increase of the Hill reaction was observed upon addition of 5 mM $NaHCO_3$, whereas at 4 Hz flash frequency the effect of $NaHCO_3$ addition on the O_2 yield in the first flashes was less than a factor of 2.

The normal flash pattern at long flash intervals and the rather large effects at saturating light intensity suggest that the inhibition in the absence of CO_2/HCO_3^- is caused by a decrease in kinetic rate constants of electron transfer from Q_A to Q_B rather than by a change in the semiquinone equilibrium between $Q_A^- \cdot Q_B$ and $Q_A \cdot Q_B^-$. In order to test whether the equilibrium Q_A^- concentration was changed, we measured the S_2 and S_3 decay in '$-CO_2$' thylakoids with and without added HCO_3^-. The results are plotted in Fig. 6. No important changes in rates of the slow phases of S_2 and S_3 decay are observed. Compared to Fig. 2, the decay, especially of S_2, is slower; this is a pH effect (see below). However, the fast phase in reduction of S_2 and S_3 is completely abolished in the absence of HCO_3^-. This indicates that the

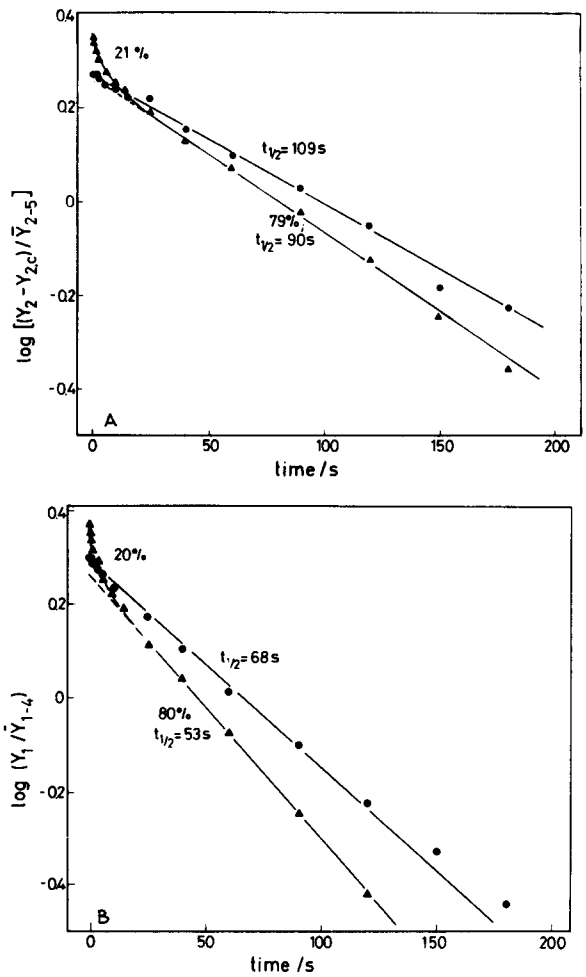

Fig. 6. Decay kinetics of the S_2 (A) and S_3 (B) states in pea thylakoids in a medium described in Fig. 5, with (▲) and without (●) the addition of 5 mM $NaHCO_3$ to both thylakoid suspension and electrode buffer.

electron transfer from the unknown donor to S_2 or S_3 is blocked in the absence of HCO_3^-. So far, only one clear effect of HCO_3^- on electron transport not involving the $Q_A \cdot Q_B$ complex has been reported: the Q_A^- oxidation by C400 (a one-electron acceptor with a midpoint potential at +400 mV) was found to be blocked in the absence of HCO_3^- [22]. C400 is not likely to be involved in the fast phase of the S_2/S_3 reduction because of its high redox midpoint potential.

In order to detect possible differences in the equilibrium Q_A^- concentration in the absence and the presence of bound HCO_3^-, the decay rate of

Q_A^- in the presence of DCMU was measured. The chlorophyll *a* fluorescence decay was found to have a half-time of 2.26 ± 0.05 s in the absence and 0.96 ± 0.03 s in the presence of 5 mM $NaHCO_3$. Thus, the back-reaction from Q_A^- to S_2 is slowed down in the absence of bound HCO_3^- by a factor of about 2.3. Since in the absence of DCMU, the S_2 decay is almost independent of HCO_3^-, this indicates that the equilibrium Q_A^- concentration in the absence of HCO_3^- has to be increased by a factor of 2.3 in order to keep the S_2 reduction rate at a constant level. However, the somewhat increased Q_A^- concentration in the absence of bound HCO_3^- cannot explain the large HCO_3^- effects on electron transport in saturating light. For this, the changes in the kinetic constants of electron transport [23] are probably most important.

The decrease in back reaction rate in the absence of bound HCO_3^- and in the presence of DCMU as observed here provides an explanation for the finding that the chlorophyll *a* fluorescence induction curve on the time-scale of 1 s in the presence of DCMU was faster in the absence of bound HCO_3^- than in its presence [24]: in the presence of HCO_3^- the back-reaction to S_2 may play an important role, whereas this is not the case in the absence of HCO_3^-.

The influence of pH

In some experiments described above, we have been able to measure effects of the equilibrium constant of the $Q_A^- \cdot Q_B / Q_A \cdot Q_B^-$ equilibrium (neglecting possible protonation of Q_B) on the rate of decay of S_2. In order to measure pH effects on the semiquinone equilibrium between Q_A and Q_B, we measured S_2 decay as well as chlorophyll *a* fluorescence decay in the presence of 5 μM DCMU. We observed that the S_2 decay in the absence of DCMU got slower upon decreasing the pH to 6.0 (the half-time of the slow phase increased from 34 s at pH 7.6 to 90 s at pH 6.0). The $t_{1/2}$ of fluorescence decay in the presence of DCMU, however, decreased from 1.15 ± 0.02 s at pH 7.6 to 0.73 ± 0.03 s at pH 6.0. Assuming that only the Q_A^- concentration and not the S_2 fraction that can react with Q_A^- (the transition $S_1 \rightarrow S_2$ is not related to proton release) is dependent upon pH, this suggests that the equilibrium Q_A^- concentration after one flash in the absence of inhibitor is decreased by a factor of $90/34 \times 1.15/0.73 = 4.2$ upon changing the pH from 7.6 to 6.0. This indicates that, if protonation of Q_B^- or of a neighboring group plays an important role in 'stabilizing' Q_B^- such that the amount of $Q_A^- \cdot Q_B$ relative to $Q_A \cdot Q_B^-(H^+)$ strongly decreases, the pK_a value of this protonation is likely to be slightly higher than 7.6. Otherwise, no pH effect ($pK_a > 8$) or an approx. $10^{1.6}$-fold (40-fold) effect ($pK_a \ll 6$ under additional assumption that the $Q_A^- \cdot Q_B \cdot H^+ / Q_A \cdot Q_B^- \cdot H^+$ ratio is almost zero) would have been expected. However, it should be noted that the estimations made above are very rough and somewhat qualitative. Recently, Crofts et al. [25] have estimated the pK_a of the protonation reaction of $Q_A \cdot Q_B^-$ to be 7.9, which fits nicely with our estimation. Experiments at many more pH values are needed in order to test the suggestions made here.

Thus, these data show that measurements of the decay kinetics of the S-states, especially of S_2, together with fluorescence decay measurements in the presence of DCMU, provide a tool for measuring the equilibrium concentration of $Q_A^- \cdot Q_B$ relative to that of $Q_A \cdot Q_B^-$.

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