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PRIMARY REACTIONS OF PHOTOSYSTEM II AT LOW pH

I. PROMPT AND DELAYED FLUORESCENCE

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SUMMARY

Prompt and delayed chlorophyll fluorescence have been studied in broken spinach chloroplasts at pH values down to 2.6. No direct effect of low pH on the primary charge separation in Photosystem II was observed. The irreversible inactivation of a secondary electron donor in a narrow pH range around pH 4.5 was demonstrated. At lower pH values the photooxidized form of a more primary electron donor, revealed by its efficient fluorescence quenching, was reduced with a half time of about 200 μs, 25 % by another electron donor and 75 % by back reaction with the reduced acceptor. The electron donation had a half time of 800 μs and was practically irreversible. The back reaction had a pH dependent half time: about 270 μs at pH 4 and increasing towards lower pH. The competition of both reactions resulted in a net efficiency of the charge separation at pH 4 of 25 %, increasing towards lower pH.

INTRODUCTION

The primary charge separation in Photosystem II is rapidly stabilized by electron transfer reactions on the donor side of the reaction center. These fast reactions can be studied in intact systems with considerable technical effort only, and much valuable information may still be expected from physically or chemically modified systems in which these reactions are slowed down. Low pH may induce this condition because the midpoint potential of the electron carriers involved may not be pH dependent while that of the water/oxygen couple certainly is.

Several studies on the effects of low pH on system II have been reported, mostly with emphasis on the relation to the light-induced acidification of the internal space of the thylakoids. Since oxygen evolution is largely inhibited already at pH 5,

Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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and also because of practical difficulties due to aggregation of chloroplasts and pheophytinization of chlorophyll, very low pH values were usually avoided. Wraight et al. [1] who studied the pH dependence of prompt and delayed fluorescence down to pH 4, observed a marked increase of 100 ms delayed fluorescence at low pH, which was attributed to the accumulation of a high S-state resulting from the inhibition of oxygen evolution. Measurements by Velthuys [2] show that the acidification inhibits the decay of high S-states, as indicated by their stimulation of 40 ms delayed fluorescence, and incubation at pH 4 induced a stimulation even with dark adapted chloroplasts.

This paper reports mainly on the influence of pH values lower than 5 on the donor side of the Photosystem II reaction center.

MATERIAL AND METHODS

Freshly prepared spinach chloroplasts in Tricine buffer (50 mM *N*-tris(hydroxymethyl)-methylglycine, 10 mM KCl, 2 mM $MgCl_2$, 400 mM sucrose, pH 7.8) were diluted just before measurement 10 to 20 times with an appropriate buffer solution without sucrose to obtain a suspension of broken chloroplasts of the desired concentration and pH. Addition of uncouplers (5 μM valinomycin and 5 μM nigericin) had no effect on the phenomena described. At the lowest pH values the chloroplasts aggregated immediately, but after homogenizing the suspension it took some time before aggregation started again. Down to pH 2.5 this time was long enough for the measurements reported here. No significant pheophytinization occurred on the time scale of these measurements. Fumarate, succinate and phosphate buffers were used in overlapping series and all phenomena reported here were checked to be independent of the particular buffer used. pH values given in the figures are final values in the sample. The pH dependencies presented in this paper varied somewhat from preparation to preparation. In unbroken chloroplasts the phenomena described appeared to be shifted slightly to lower pH.

Prompt and delayed fluorescence were measured through a filter combination transmitting a broad band around 680 nm, with blue actinic and measuring light (Corning CS 4-96). In most experiments the emission excited by the actinic light was measured; only in the experiments of Figs. 1 and 5 was a separate weak measuring beam used. Illumination and measurement were on the same side of a 1 mm cuvette, with a chlorophyll concentration of 25 to 50 μg/ml. The measurement of fluorescence yield kinetics during a flash was carried out as described in ref. 3.

Decay curves of delayed fluorescence were measured both with a rotating disc phosphoroscope as described in ref. 4, modified to give a 1 ms light–9 ms dark cycle, a peak illumination intensity of about 100 mW/cm^2, and a 10 μs time resolution, and with the fast laser phosphoroscope of Lavorel [5] modified as follows: the light source was a 10 mW Helium Cadmium laser (Liconix 401 M) emitting at 441.6 nm, chopped at 20 Hz (250 μs light period) with an electronically triggered acousto-optical modulator (rise time 400 ns) using a quartz stabilized oscillator as the time base. The same apparatus was used for the measurements of fluorescence induction at high light intensity. All measurements were carried out at room temperature.

RESULTS

Prompt fluorescence

The minimal chlorophyll fluorescence yield, measured with an excitation intensity weak enough to have no actinic effect and in the presence of a low ferricyanide concentration was largely independent of pH (Fig. 1, solid circles). In the absence of ferricyanide the fluorescence yield was higher below pH 4.0 (Fig. 1, squares) suggesting that the primary acceptor may be partially in the reduced form in the dark. The maximal fluorescence yield, which could be obtained either by strong illumination in the presence of DCMU, or chemically by addition of dithionite, gradually decreased below pH 5, as was reported also in ref. 1 (Fig. 1, open circles). This decrease of the maximal yield was not always as pronounced as shown here. It could not be prevented by addition of $MgCl_2$. Ferricyanide, at a concentration of 1 mM or more, completely abolished the light-induced fluorescence rise below pH 4 even in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) while at pH 5 almost the maximal yield could still be obtained (Fig. 1, triangles).

The same narrow transition region in the pH dependence was observed also in the fluorescence rise kinetics in continuous light in the presence of DCMU. Because of the linear relation between variable fluorescence yield and rate of electron transport [6, 7] the area over the fluorescence rise curve is proportional to the pool size of system II electron acceptors [8]. In the presence of DCMU, which permits the photoreduction of the primary acceptor only, the area over the full rise curve should be proportional to the quantum requirement if the absorbed light intensity is kept constant. Fig. 2 shows the pH dependence of the reciprocal of this area, measured in the presence of DCMU and a ferricyanide concentration (5 μM) which permitted the measurement of the full rise curve from minimal to maximal level. In order to correct for the pH dependence of non-photochemical quenching the rise curves were normalized to give the same maximal fluorescence increase. As the decrease of the maximal

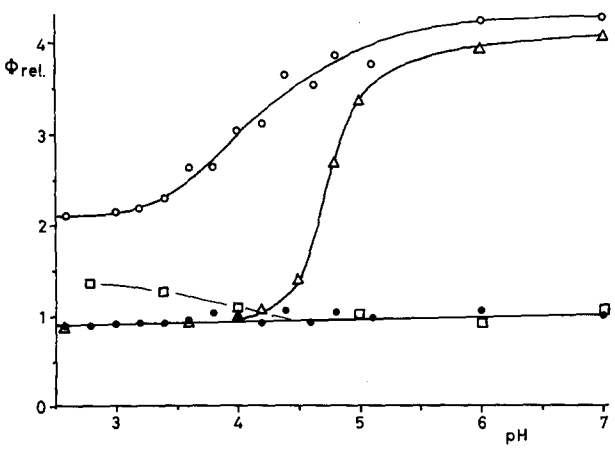

Fig. 1. pH dependence of fluorescence yield. □—□, dark, no additions; ●—●, dark, 10 μM ferricyanide; ○—○, dark with dithionite, the yield obtained by illumination in the presence of 10 μM DCMU was not significantly different; △—△, strong light (100 mW/cm^2), 1 mM ferricyanide, 10 μM DCMU.

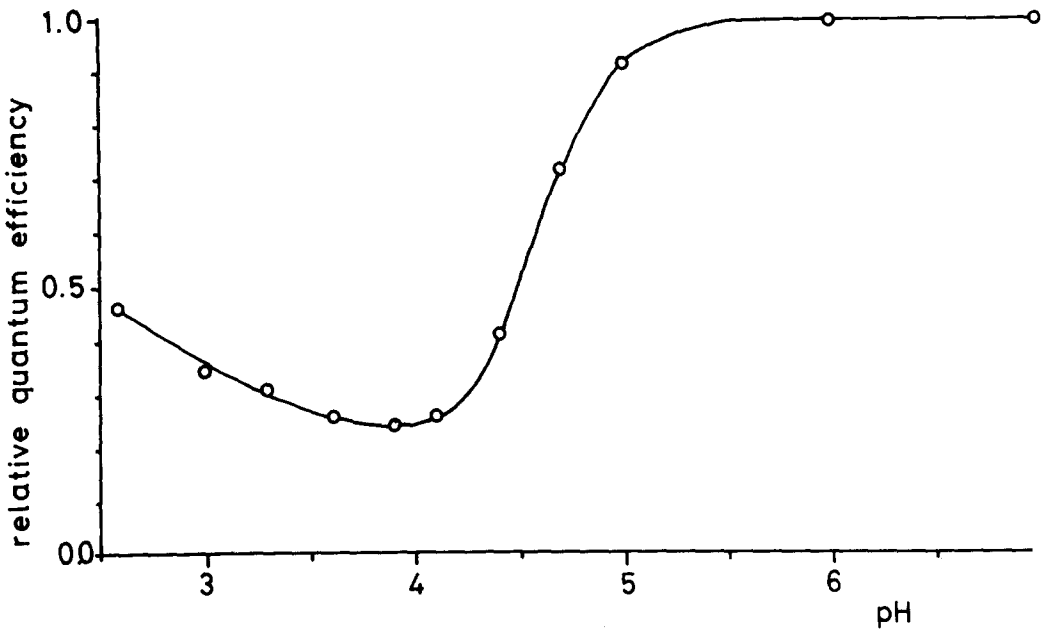

Fig. 2. pH dependence of the relative quantum yield of the light-induced fluorescence rise in the presence of DCMU. Efficiency calculated as the reciprocal of the deficit area over the complete fluorescence rise curve, after normalization of the maximal fluorescence increase at different pH values. Additions: 5 μM ferricyanide, 10 μM DCMU. Absorbed light intensity 8.7 nE $\cdot$ cm^{-2} $\cdot$ s^{-1}.

fluorescence yield at low pH could have changed the linearity between variable fluorescence and electron transport, this correction is not a priori allowed, but it is justified by flash experiments described below. The quantum efficiency decreased four-fold on lowering the pH from 5 to 4 and at lower pH gradually increased again. It was checked that DCMU has its normal inhibitory effect on the dark decay of the fluorescence yield in the whole pH range. Below pH 4 the dark decay was extremely slow (in the order of 15 min.).

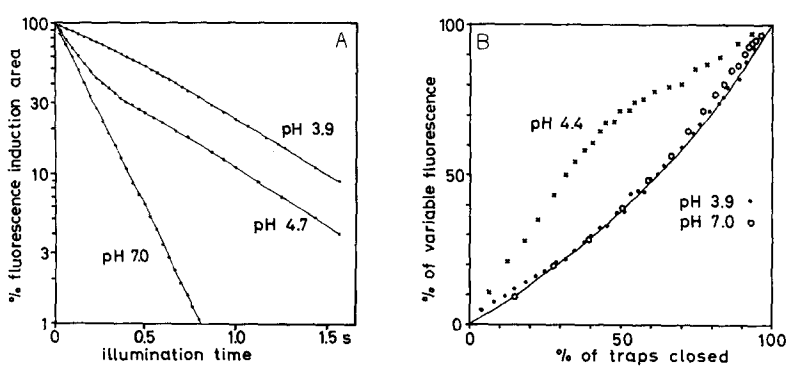

Fig. 3. Time course of the decrease of the deficit area (A) and relation of variable fluorescence yield to deficit area (B) during fluorescence induction. Conditions as in Fig. 2.

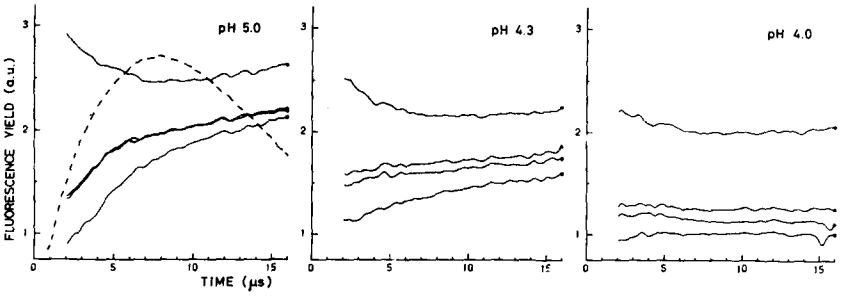

Fig. 4. Fluorescence yield measurements during a saturating 16 μs flash in the presence of DCMU. The four curves at each pH represent, from the bottom upwards, the first three flashes of a series (spaced at 2.5 s) and one flash given during continuous illumination. - - - represents the flash intensity at an arbitrary scale.

Below pH 4 and above pH 5 the fluorescence induction curves in the presence of DCMU indicated a rather homogeneous behaviour of the reaction centers, as shown by the time course of the decrease of the number of open reaction centers (Fig. 3A). The kinetics were complicated mainly by the non-linear relation of electron transport rate to the number of open centers (Fig. 3B) which may be attributed to energy transfer between photosynthetic units [9]. At pH values between 4 and 5 the fluorescence induction curves were clearly biphasic, indicating a heterogeneity among reaction centers. This shows that the decrease in efficiency upon lowering the pH from 5 to 4 was due to an abrupt, qualitative change in the reaction centers. The efficiency could not be restored by increasing the pH.

The same conclusion may be drawn from the flash experiments shown in Fig. 4. The extent of the fast fluorescence rise during a 16 μs flash decreased sharply to zero between pH 5 and pH 4. Below pH 4 no significant increase occurred during the flash but the fluorescence yield in a second flash was higher. By varying the dark time between the two flashes the half time of the fluorescence rise was found to be a

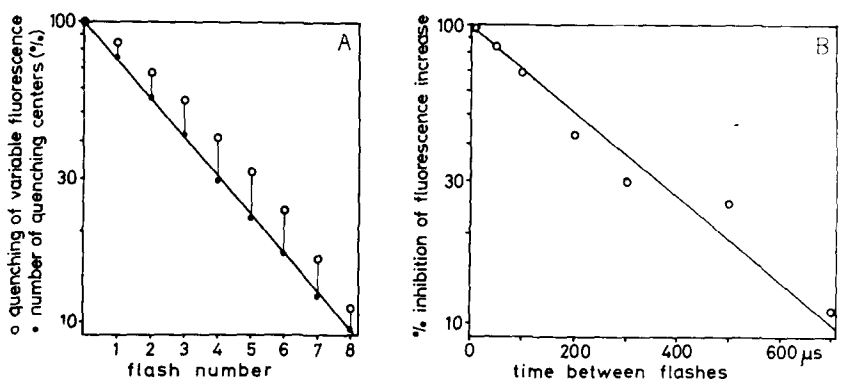

Fig. 5. Flash-induced fluorescence yield increase at pH 3.9 in the presence of DCMU, measured with a very weak excitation beam. The photomultiplier was closed during the flash. A, decrease of the number of open traps during a flash series (● — ●), obtained from the decrease of variable fluorescence quenching (○ — ○) with the aid of Fig. 3B. The results were independent of the spacing between flashes in the range from 10 s to 1 ms. Shorter dark times between two flashes caused an inhibition of the fluorescence increase induced by the second flash (B).

few hundred μs. The dark decay was very slow, as noted already in the measurements of Fig. 2, and the maximal yield could be obtained by ten to fifteen flashes. After correction for energy transfer between units with Fig. 3B each flash was found to close about 25 % of the open reaction centers, as shown in Fig. 5A. This value seems to confirm the quantum efficiency measurements of Fig. 2.

The flash yield of 25 % was not due to a low efficiency of the photochemical reaction: the flashes were completely saturating and a second flash, given immediately after the first one, did not cause an additional fluorescence increase. Apparently the first flash did activate all available reaction centers. The expected fluorescence increase due to the reduction of the primary acceptor was observed only after hundreds of microseconds, however, so the transient presence of another efficient fluorescence quencher must be assumed. The disappearance of this quencher left only 25 % of the centers in a high fluorescent, inactive state. The remaining 75 % was evidently reopened, presumably by back reaction because the presence of DCMU would not allow reoxidation of the primary acceptor otherwise. The reopening of these centers had (at pH 3.9) a half time of 200 μs (Fig. 5B), similar to the half time of the fluorescence increase after the flash.

As a minimal model one might assume that both reactions, back reaction and stabilization in high fluorescent state, are competitive decay routes of the transient fluorescence quenching state formed by the flash. If so the sum and the ratio of both reactions are known and lead to the conclusion that at pH 4 the back reaction should have a half time of 270 μs and the stabilization reaction 800 μs.

Upon continuous illumination with a light intensity high enough to completely saturate this back reaction a slow fluorescence rise was observed (Fig. 6A). After correction for energy transfer using the data of Fig. 3B a half time of 800 μs was indeed found for the stabilization of reaction centers in a high fluorescent state (Fig. 6B). The 800 μs rise could be observed up to pH 4.6 and did not seem to be pH dependent. Between pH 4 and pH 5 the 800 μs rise was replaced by the very fast rise as illustrated already in Fig. 4. As shown in Fig. 1 the slow fluorescence rise was abolished completely by 1 mM ferricyanide, which had no effect on the fast rise. Up to pH

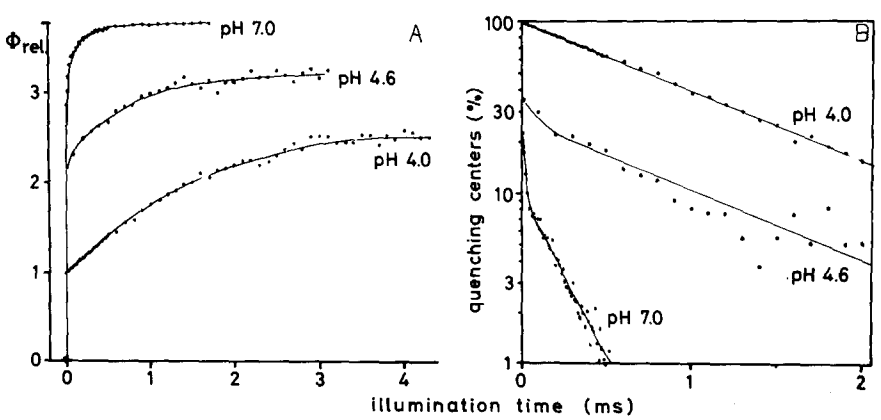

Fig. 6. Fluorescence rise curves upon very strong continuous illumination (A) and decay kinetics of the low fluorescent state corrected for energy transfer (B).

5 the omission of DCMU did not change these fluorescence induction curves. At higher pH values the complicated kinetics described by Delosme [10] were observed.

Delayed fluorescence

Decay curves of delayed fluorescence in the sub-millisecond and millisecond range after strong illumination were measured with a conventional rotating disc phosphoroscope and with a fast laser phosphoroscope. It should be kept in mind that this method selectively enhances decay components extending over more than one light-dark cycle and that it tends to obscure very fast components which decay within the illumination time. Typical decay curves are shown in Fig. 7. Down to pH 5 the decay was highly polyphasic. The most rapid decay component observed in the conventional phosphoroscope had a pH independent half time of somewhat less than 100 μs and the laser phosphoroscope revealed in addition a 25 μs component at neutral pH. At pH 4 and below the first decay component had a slightly pH dependent half time around 200 μs. At pH 4 the contribution of slower components was minimal and the 200 μs component accounted for more than 90 % of the decay. At pH values between 4 and 5 the decay may have consisted of contributions from both the 100 μs and the 200 μs components, but these times were too close to distinguish them. As shown in Fig. 8A ferricyanide or DCMU had little effect on these decay times. The 200 μs component may be slowed down by DCMU and below pH 4 also by ferricyanide, but only with both additions the increase of the decay time was clearly significant. The 100 μs decay time was not significantly changed by additions, but its determination was less reliable anyway, because of the uncertainty in subtracting the large and polyphasic slower components. Obviously the transition between pH 4 and 5 from the 200 μs to the 100 μs decay and also the 200 μs decay time itself suggest a correlation with the results on prompt fluorescence described above.

The pH dependence of the amplitude of the fast decay components is shown in Fig. 8B. The 200 μs component was maximal near pH 4 and the 100 μs component

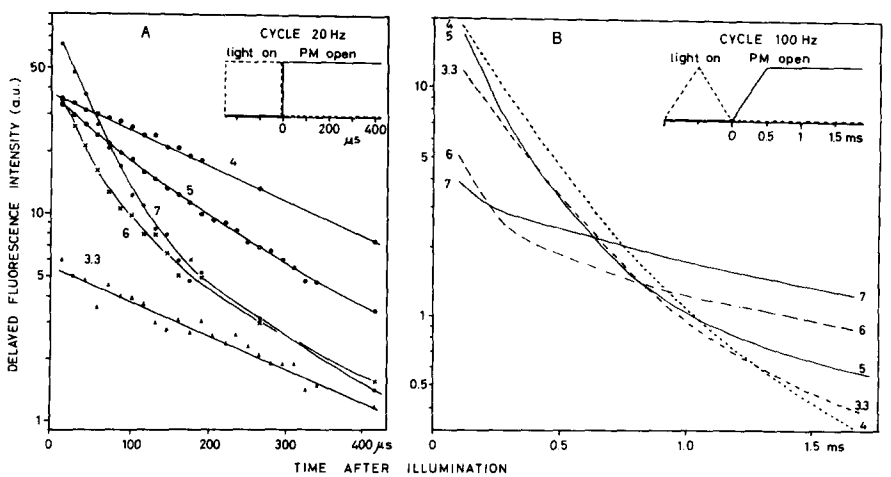

Fig. 7. Decay kinetics of delayed fluorescence measured with a fast laser phosphoroscope (A) and with a rotating disc phosphoroscope (B) as described in Methods. Each curve is the average of 2500 decays in A and 512 decays in B. No additions.

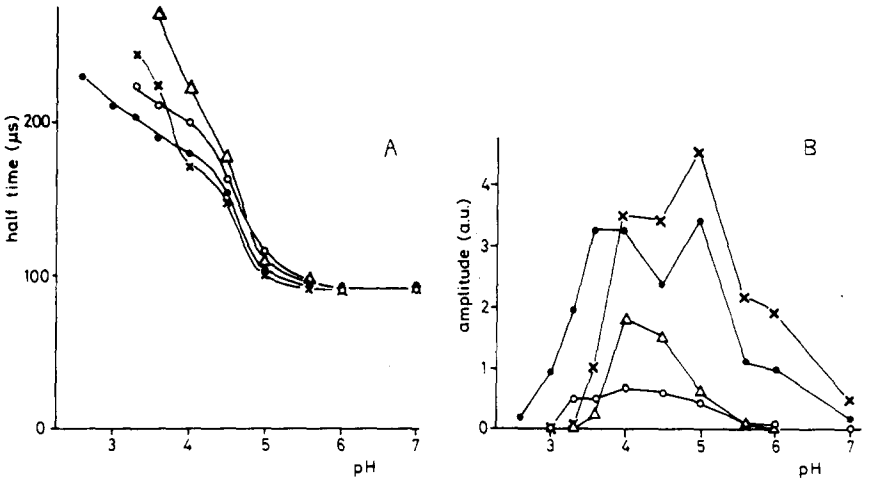

Fig. 8. pH dependence of the half time (A) and amplitude (B) of the most rapid component observed in the decay of delayed fluorescence, measured as in Fig. 7B. ●—●, no additions; ○—○, 10 μM DCMU; ×—×, 1 mM ferricyanide; △—△, 1 mM ferricyanide and 10 μM DCMU.

near pH 5 whether DCMU or ferricyanide or both were present or not. DCMU decreased the amplitude of both components, which was due to accumulation of closed reaction centers because the reoxidation of the reduced acceptor became much slower than the light-dark cycle. The same explanation applies to the increased amplitude of the 100 μs component in the presence of ferricyanide, because in its absence the illumination was intense enough to keep the endogenous acceptor pool largely in the reduced state. The effect of ferricyanide on the 200 μs component was pH dependent: at pH 4 it partially relieved the DCMU inhibition but had little effect by itself; at lower pH it strongly inhibited delayed fluorescence. At pH 3 and below the emission intensity was decreased by at least three orders of magnitude (providing an ideal check for false light in the apparatus). The decrease was not due to a large change in the decay time (Fig. 8A) so it reflects either a decrease of the number of participating reaction centers or a decrease of the "exciton yield" of the back reaction.

DISCUSSION

The results reported in this paper revealed no pH dependence of the photochemical reaction in system II from pH 7 down to pH 2.6. Secondary electron transport is inhibited at low pH in several ways. The reoxidation of the primary acceptor is slow and incomplete at low pH values and the minimal fluorescence yield is obtained only by addition of ferricyanide. The very fast fluorescence increase disappears irreversibly between pH 5 and pH 4. This is an abrupt, qualitative change, because the fluorescence kinetics near pH 4.5 are heterogeneous rather than intermediate between those at pH 5 and at pH 4. Fluorescence quenching by an oxidized donor in the Photosystem II reaction center now seems established [11–14]. This component, which completely quenches the variable fluorescence, is presumably reduced within a few microseconds by a non-quenching secondary donor [13]. At pH 4 the fast secondary donor is inactivated and the life time of the quencher at the donor side is

greatly increased. It has a half life of 200 μs, which is determined by two competing processes: a 270 μs back reaction and an 800 μs electron donation, the latter being accompanied by an increase of the fluorescence yield which is reversible only on a time scale of minutes. This competition results in a 25 % efficiency of the fluorescence rise induced by weak continuous or saturating flash light. If the back reaction is the source of delayed fluorescence the evidence from fluorescence measurements is supported by the pronounced 200 μs decay of delayed fluorescence and the minimum of slower components observed at pH 4. At still lower pH values the 800 μs electron donation remains unchanged but the efficiency of the fluorescence rise and the decay time of delayed fluorescence are increased, indicating a slowing down of the back reaction.

The effects of ferricyanide are remarkable. Ferricyanide prevented the light-induced fluorescence rise at pH 4 and below, even in the presence of DCMU. In the minimal model, derived from flash experiments on prompt fluorescence, this effect of ferricyanide can be explained either by a rapid reoxidation of the primary acceptor, implying a bypass of the DCMU block, or by an inhibition of the 800 μs electron donation. Both explanations predict opposite effects on the decay time of delayed fluorescence : a rapid reoxidation of the acceptor should accelerate the decay of delayed fluorescence, while an inhibition of the 800 μs electron donation should result in a 270 μs half time of delayed fluorescence. Actually, ferricyanide had no pronounced effect on the decay time of delayed fluorescence in the presence of DCMU at pH 4. This leads to the conclusion that both effects of ferricyanide occur simultaneously and nearly cancel each other.

The oxidation of the acceptor and the inhibition of electron donation occurring together might result in a light-induced accumulation of the oxidized form of the primary donor. Absorbance changes with the difference spectrum of *P* 680 could actually be measured in this way [15]. At pH values below 4 the sharp decline of the amplitude of delayed fluorescence suggest that near pH 3 it is even possible to block all reaction centers in a state where both the acceptor and the donor are in the oxidized form.

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