Biochimica et Biophysica Acta, 440 (1976) 98–106

BBA 47126

PRIMARY REACTIONS OF PHOTOSYSTEM II AT LOW pH

2. LIGHT-INDUCED CHANGES OF ABSORBANCE AND ELECTRON SPIN RESONANCE IN SPINACH CHLOROPLASTS

MARTIN P. J. PULLES, HANS J. VAN GORKOM and GERARD A. M. VERSCHOOR

Department of Biophysics, Huygens Laboratory, Wassenaarseweg 78, Leiden (The Netherlands)

(Received December 29th, 1975)

SUMMARY

The effects of lowering the pH on Photosystem II have been studied by measuring changes in absorbance and electron spin resonance in spinach chloroplasts.

At pH values around 4 a light-induced dark-reversible chlorophyll oxidation by Photosystem II was observed. This chlorophyll is presumably the primary electron donor of system II. At pH values between 5 and 4 steady state illumination induced an ESR signal, similar in shape and amplitude to signal II, which was rapidly reversed in the dark. This may reflect the accumulation of the oxidized secondary donor upon inhibition of oxygen evolution. Near pH 4 the rapidly reversible signal and the stable and slowly decaying components of signal II disappeared irreversibly concomitant with the release of bound manganese.

The results are discussed in relation to the effects of low pH on prompt and delayed fluorescence reported earlier (van Gorkom, H. J., Pulles, M. P. J., Haveman, J. and den Haan, G. A. (1976) Biochim. Biophys. Acta 423, 217–226).

INTRODUCTION

We recently reported measurements of prompt and delayed fluorescence at low pH [1]. The results could be explained by a model in which, after the charge separation in Photosystem II, a back reaction with a halftime of a few hundred μs competes with a secondary electron donation. At neutral pH this donation occurs very fast (halftime $\leqslant$ 1 μs, cf. ref. 2), but below pH 4.5 it is replaced by a much slower one with a halftime of 800 μs. In this model both the primary acceptor and the primary donor quench fluorescence when they are in the oxidized state. The model implies that at pH 4 the reduction of the oxidized primary donor may become slower than the oxidation of the reduced primary acceptor, especially when an oxidant like ferricyanide is present. Under these circumstances accumulation of the oxidized primary donor should be possible in continuous light in the steady state. Changes of absorbance and electron spin resonance induced by strong illumination at low pH,

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

indeed appeared to confirm the steady state oxidation of the reaction center chlorophyll of Photosystem II, *P*-680. These changes, which may be ascribed to the oxidation of a chlorophyll *a* dimer [3], have been observed in purified system II particles [4]. In addition we have studied the inactivation of secondary electron donation near pH 4.5. Our results support the assignment of a rapidly decaying component of ESR signal II to the secondary donor [5].

METHODS

Chloroplast preparation and buffers used are described in ref. 1. ESR measurements were performed with a Varian E9 spectrometer as described in ref. 4. Absorbance measurements were done in a split-beam apparatus similar to the one described in ref. 6. The chlorophyll concentration was determined by the method of Arnon [7].

To correct the absorbance measurements for the drastic effects caused by particle flattening (or sieve effect, ref. 8) the following equation, obtained by Amesz [6], was used:

$$E'(\lambda) = p \log e(1-T'_{\mathrm{p,av}}(\lambda)) \tag{1}$$

where E' is the absorbance of the suspension, corrected for scattering, p is the projected area of all particles on a plane perpendicular to the measuring beam per cm^2, e is the base of the natural logarithm and $T'_{\mathrm{p,av}}$ is the averaged transmitance of one particle. Scattering corrections were performed according to the method of Latimer and Eubanks [9]. Fig. 1 (adapted from ref. 6) gives the relation between the averaged transmittance of a sphere and the flattening factor E_{sol}/E' (where E_{sol} is the absorbance of the same pigments in solution) and the differential flattening factor (i.e. the flattening factor obtained for small absorption changes) $\Delta E_{\mathrm{sol}}/\Delta E'$. When the absorbance spectrum of the suspension is known it is possible to obtain the flattening correction at all wavelengths with the aid of Eqn. 1 and Fig. 1, provided that the proportionality constant p is known. Knowledge of the flattening factor at one wavelength permits the calculation of p. The flattening factor in the blue absorption maximum of spinach

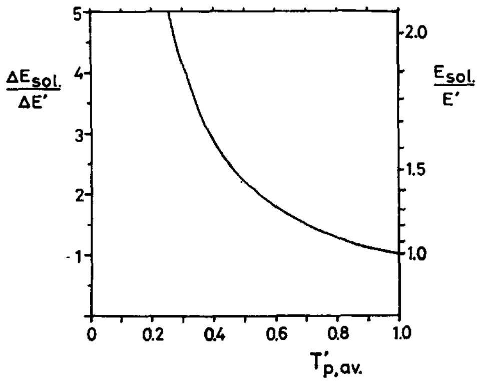

Fig. 1. Flattening factor (E_{sol}/E') and differential flattening factor ($\Delta E_{\mathrm{sol}}/\Delta E'$) as a function of the averaged transmission of one spherical particle, corrected for scattering ($T'_{\mathrm{p,av}}$), adapted from ref. 6. Further explanation, see Methods.

chloroplasts was determined by extraction with ethanol and was found to be 1.77, in very good agreement with the results of Latimer and Eubanks [9], who calculated the flattening correction by estimating *p* microscopically.

RESULTS

Strong illumination of a chloroplast suspension at pH 4 caused a fast bleaching near 680 nm, which was rapidly reversed in the dark. Recorder tracings of this and several control experiments are shown in Fig. 2. Addition of ferricyanide increased the extent of the 680 nm change and in some preparations its presence was required to see it at all. The change was abolished by DCMU, although only partly so in the presence of ferricyanide, indicating that more than one turnover per reaction center is needed. Interference by *P*-700 could be excluded because at low pH ferricyanide kept *P*-700 in the oxidized form. It was checked that *P*-700 did not contribute significantly to the absorbance change at 680 nm and that the effect of fluorescence excited by the measuring beam was negligible as well.

The spectra of the light-induced absorbance changes at pH 4 in the presence of 1 mM ferricyanide and in the presence of 5 μM DCMU are shown in Fig. 3 (open circles and open squares respectively). In the presence of ferricyanide the spectrum, after correction for particle flattening (see Methods, solid circles), resembles very much the spectrum measured in small Photosystem II particles under the same conditions [4], which was attributed to the oxidation of *P*-680. However, there are some differences: the main band near 680 nm occurs at a few nm longer wavelengths than

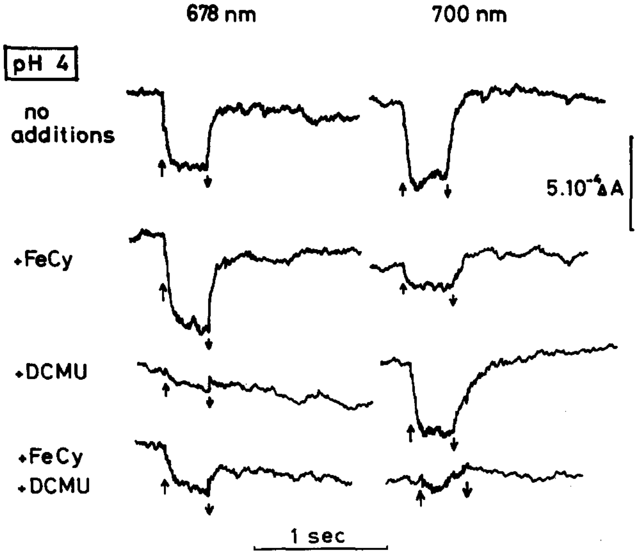

Fig. 2. Light-induced absorbance change at 678 and 700 nm under different conditions as indicated. Upward and downward arrows indicate light on and light off respectively. Each trace is the average of five measurements. Optical path length 1.2 mm, halfband width 3 nm. Actinic illumination: 300 mW · cm^{-2} blue light (Corning CS 4-96). Chlorophyll concentration: 110 μM. Ferricyanide (FeCy) concentration: 1 mM. DCMU concentration: 5 μM.

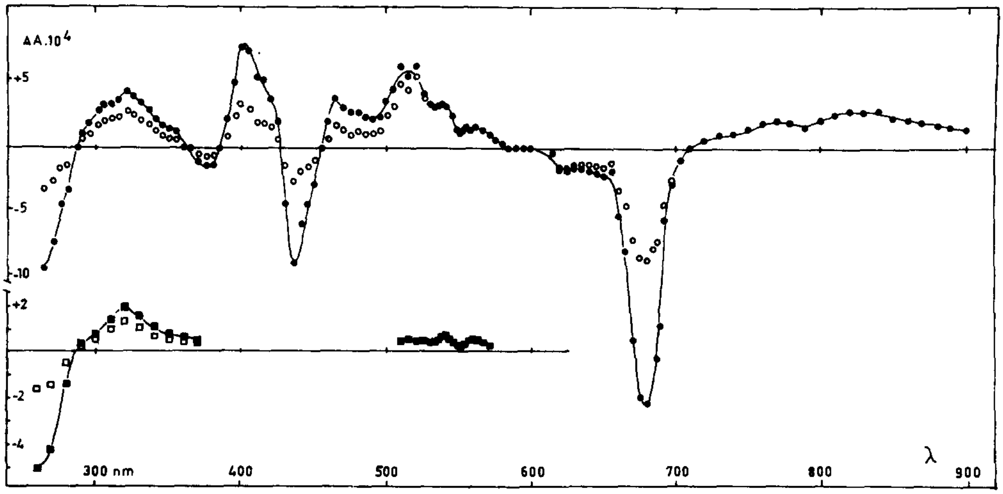

Fig. 3. Light minus dark absorbance difference spectra at pH 4. Circles: in the presence of 1 mM ferricyanide, squares: in the presence of 5 μM DCMU. Solid symbols indicate the absorbance changes corrected for particle flattening as indicated in Methods; where this correction was significant, the measured values are shown as well (open symbols). Chlorophyll concentration 110 μM. A satisfactory signal-to-noise ratio was obtained by averaging (from 3 measurements in the near infrared up to 40 in the ultraviolet). A new sample was taken every 5–20 measurements, gradual inactivation was corrected for. Optical path length 1.2 mm; bandwidth adjusted to the wavelength interval between measurements. Absorbed actinic intensity about 500 nE · $cm^{-2} \cdot s^{-1}$ (red or blue, adjusted to obtain the same actinic effect). Blue and red regions of the spectrum were calibrated to each other in separate measurements with green illumination.

in the system II particles, additional absorbance increases near 515 and 420 nm can be seen, and there is probably a contribution of the reduction of the primary acceptor, as suggested by the spectrum in the 550 nm region (*C*-550), and in the near ultraviolet. Although the spectrum in the presence of 5 μM DCMU (squares in Fig. 3) contains other components, such as the oxidation of *P*-700 (see Fig. 2), the reduction of the primary acceptor is evident. In the green region the bandshift of C-550 is visible; in the near ultraviolet the spectrum suggests the reduction of plastoquinone to its semiquinone anion, although the absorbance decrease at 260 nm is too large relative to the increase at 320 nm, indicating some additional absorbance decrease in this region of the spectrum. The two times smaller amplitude of the ultraviolet changes relative to those measured in the presence of ferricyanide is due to incomplete reoxidation of Q^- between light periods.

Changes in ESR at pH 4, induced by strong illumination in the presence of 8 mM ferricyanide are shown in Fig. 4A. In these experiments a flow system was used, which, approx. 1 min before the measurement, diluted a chloroplast suspension 5 times with a buffer solution of pH 4, containing ferricyanide. As is indicated in the inset, the dark decay at 3399 G seems to be biphasic, the fast phase was limited by the response time of the apparatus. At 3385 G only the slow phase seems to be present. The kinetic difference however was often less clear and hardly significant. The spectrum of the slow phase is broader (ΔH_{pp} = 15–20 G) than the spectrum of the fast phase (ΔH_{pp} = 7 G). Similar spectra, but with a better signal-to-noise ratio, were obtained

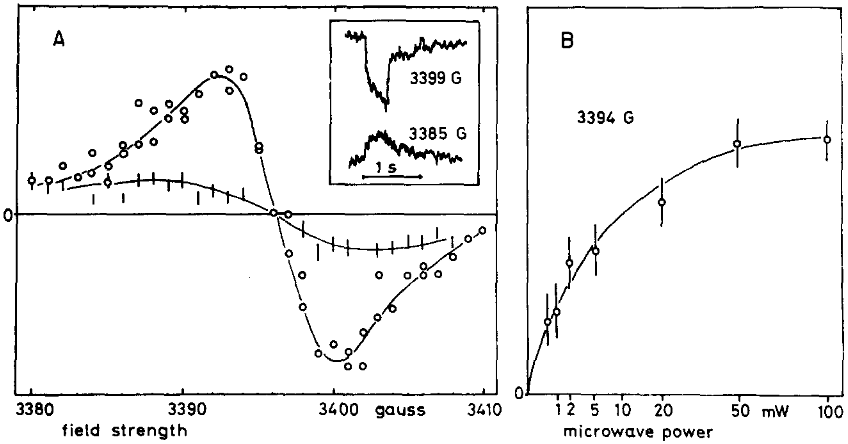

Fig. 4. (A) Light-induced ESR spectrum in the presence of 8 mM ferricyanide at pH 4. Open symbols: total change; bars: slow phase. Each symbol is the average of 50 kinetic curves with a fresh sample. Inset: kinetics at 3399 and 3385 gauss on a 2-times decreased sensitivity. Apparatus settings: microwave power 50 mW, modulation amplitude 2.5 G, time constant 30 ms, chlorophyll concentration 275 μM. (B) Power saturation curve of the light-induced change at 3394 Gauss (microwave power plotted on square root scale).

in small particles enriched in Photosystem II, and ascribed to a secondary and the primary electron donor respectively [4]. The power saturation of the rapidly decaying signal near 50 mW, shown in Fig. 4B, is compatible with the identification of this signal as an oxidized chlorophyll.

Both the ESR and the absorbance measurements show that illumination at pH 4 in the presence of a high ferricyanide concentration causes the oxidation of a chlorophyll, which is probably dimeric [3]. The DCMU sensitivity (Fig. 2) and the occurrence of the same changes in small particles devoid of Photosystem I [4] show

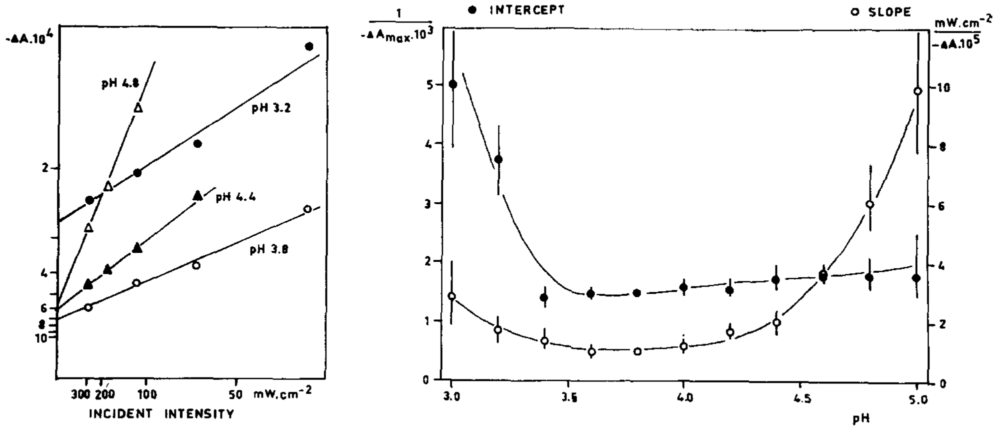

Fig. 5. (A) Double reciprocal plots of the absorbance decrease at 678 nm vs. light intensity in the presence of 1 mM ferricyanide at different pH values. (B) pH Dependence of intercept and slope of curves like those in Fig. 4A. Further conditions as in Fig. 3.

that it is a photosystem II reaction. It seems reasonble to suppose that the oxidizable chlorophyll is the primary electron donor P-680. Recently we reported [1], that under these conditions the fluorescence increase was inhibited, in agreement with earlier proposals that P-680$^+$ is a fluorescence quencher [2, 10].

In Fig. 5A double reciprocal plots are given of the steady state concentration of oxidized P-680 in the presence of 1 mM ferricyanide as a function of light intensity at different pH values. Fig. 5B summarizes this result for other pH values also. It is seen that above pH 3.5 an approximately constant amount of oxidized P-680 can be accumulated at infinite light intensity, as indicated by the intercept with the vertical axis. Using an extinction coefficient at 680 nm of 75 $\text{mM}^{-1} \cdot \text{cm}^{-1}$ and a differential flattening factor of 2.3 the maximal amount of photooxidized P-680 was calculated to be 1 per 550 chlorophyll molecules. Van Gorkom et al. [3] estimated the system II reaction center concentration at 1 per 360 chlorophylls. So under our conditions steady state oxidation of P-680 could be obtained in up to two-thirds of the reaction centers. Presumbaly in the remaining centers both the donor and the acceptor are in the reduced state, the concentration of these centers being determined by the relative relaxation rates of donor and acceptor. Below pH 3.5 however the amount of P-680 which can be oxidized strongly decreases, indicating an inactivation of the reaction centers. At pH values above 4.5 a higher light intensity is required for accumulation of P-680$^+$, which suggests that the rereduction of P-680$^+$ by secondary electron donation is becoming faster. The abrupt inactivation of the secondary electron donor near pH 4.5 as demonstrated in ref. 1 would be expected to change the intercept rather than the slope, so this effect does not seem to explain the altered intensity dependence of the steady state P-680$^+$ concentration. In agreement with the DCMU sensitivity (Fig. 2), apparently more than one turnover is required before P-680$^+$ is accumulated and its intensity dependence between pH 4 and 5 is determined not by the secondary but by a more distant electron donor. In this pH region a light-induced steady state oxidation of the secondary donor should therefore be observed.

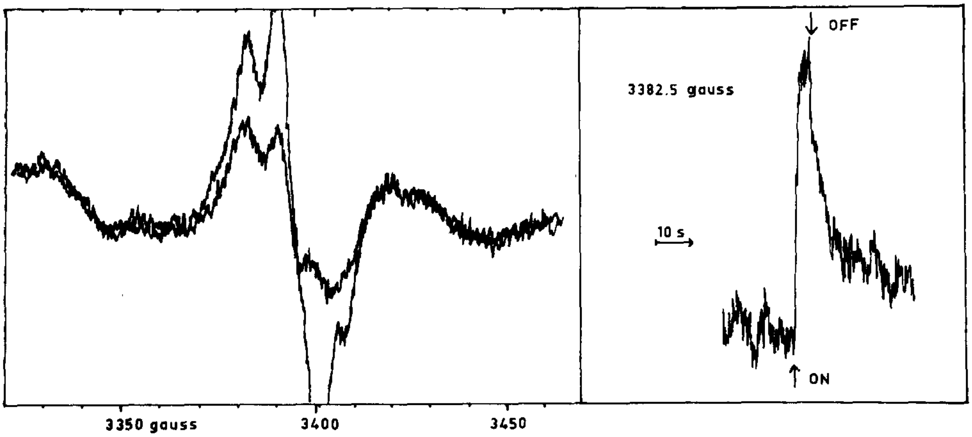

Fig. 6. ESR spectra at pH 4.6 in the dark and during red illumination and kinetics at 3382.5 G. Light intensity: 75 mW, $\lambda > 645$ nm. Chlorophyll concentration 3.5 mM. Instrument settings: microwave power 1 mW; modulation amplitude 5 G; time constant: 3 s and 1 s for spectra and kinetics respectively; scan rate 50 G/min.

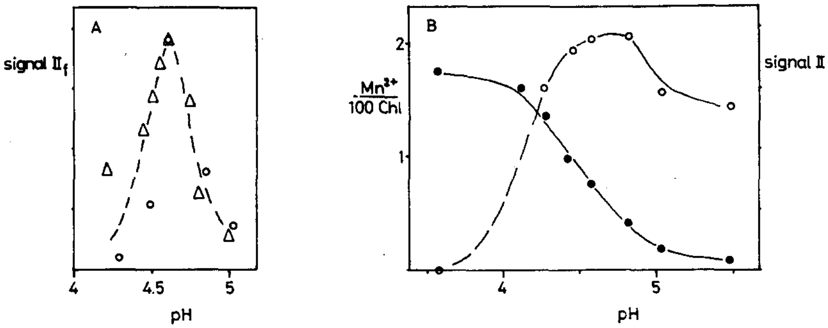

Fig. 7. pH Dependence of A, the light-induced signal II, triangles and circles correspond to different batches of chloroplasts and B, signal II present in the dark (open circles) and Mn^{2+} (solid circles). Signal II and signal II$_f$: conditions as in Fig. 6. Mn^{2+} was measured as the averaged amplitude of the 6 ESR bands of free Mn^{2+} [12]. Instrument settings microwave power: 100 mW, modulation amplitude: 10 Gauss. Mn^{2+} concentration was estimated by comparison with $MnCl_2$ solutions of known concentration. Chlorophyll concentration: 3.5 mM.

Blankenship et al. [5] have assigned a very fast decaying component of ESR signal II to the oxidized form of a secondary donor. As shown in Fig. 6 signal II could almost be doubled by illumination at pH 4.6. The light-induced increase was reversed in several seconds upon darkening. Fig. 7A illustrates that this light-induced component was observed only in a narrow pH region between pH 4 and 5. Its disappearance at the low pH side and the disappearance of the stable signal II at still lower pH were irreversible. In the same pH region bound manganese was released to a maximal amount of about 6 Mn^{2+} per reaction center (Fig. 7B), as indicated by the appearance of the ESR signal of hydrated Mn^{2+} free in solution (cf. ref. 11). The pH dependencies of these phenomena seemed to be shifted somewhat to lower or higher pH in different batches of chloroplasts, as was noted earlier for the inactivation of the secondary donor observed in fluorescence measurements [1]. The release of manganese and both signal II components were correlated in these variations. Such a correlation was also suggested by the effect of prolonged illumination (minutes) at pH values between 4.5 and 5, which caused a release of manganese and a concomitant inactivation of the light-induced signal II. This photoinactivation was apparently unrelated to photosynthetic electron transport itself, since its quantum efficiency seemed to be much higher in blue light than in red.

DISCUSSION

The results reported here show that upon strong illumination at pH values below 5, Photosystem II oxidizes a, probably dimeric (see also ref. 3), chlorophyll *a*, which is rapdily reduced again upon darkening. The most simple interpretation is that this chlorophyll is the primary electron donor in Photosystem II, *P*-680. This interpretation is consistent with the effects of low pH on prompt and delayed fluorescence, described earlier [1]. However, secondary electron donation at low temperature in the presence of ferricyanide has been ascribed to a chlorophyll dimer as well [13]. The chlorophyll oxidation described here was not strictly dependent on the presence of ferricyanide, but its identification with the primary electron donor *P*-680 must nevertheless be considered preliminary. If it is a secondary donor, one might expect to observe a decay component in delayed fluorescence accompanying its oxidation after a flash, which has as yet not been measured. Near pH 4 there is in fact a close correspondence between the decay of the delayed fluorescence, the fluorescence rise [1] and the decay of the chlorophyll cation as measured at 820 nm (Haveman, J. and Mathis, P., personal communication). The available data thus indicate that at pH 4 the oxidized primary donor has a lifetime of more then 100 μs, can be identified as an oxidized chlorophyll *a* dimer, and is an efficient fluorescence quencher.

Between pH 4 and 5 the steady state accumulation of photooxidized *P*-680 becomes more and more difficult to saturate, but the maximal amount, estimated by extrapolation to infinite light intensity does not decrease abruptly. This may not be inconsistent with the abrupt inactivation of the secondary donor in the same pH region [1] if several turnovers are required and electron donation to the secondary donor, becoming faster towards pH 5, limits the reduction of P-680$^+$. Electron transport from water is in fact largely inhibited even at pH 5 [14] and the expected light-induced accumulation of the oxidized secondary donor between pH 4 and 5 seems to be confirmed by our measurement on ESR signal II. Blankenship et al. [5] demonstrated the occurrence of a rapid transient ESR signal, with a spectrum similar to the well known signal II, during normal Photosystem II turnover and attributed this signal to the oxidized form of the physiological donor Z to P-680$^+$. We have shown [1] that the secondary donor, which normally reduced P-680$^+$ within a few microseconds, is irreversibly inactivated near pH 4.5 and the *P*-680 measurements reported here predict its steady state accumulation upon illumination at pH values between 4 and 5. Our finding that an ESR signal with the spectrum of signal II does in fact fulfill these requirements, seems to confirm the conclusion of Blankenship et al.

The irreversible disappearance of both this light-induced and of the very slowly decaying and stable forms of signal II was found to be correlated with the release of bound manganese. The solubilization of manganese at low pH may be due to its replacement by protons and seems to provide a reasonable explanation for the effects of very low pH values on the oxidizing side of Photosystem II.

ACKNOWLEDGEMENTS

Thanks are due to Irma K. van der Linden for skilful technical assistance, to Drs. J. Haveman and P. Mathis for stimulating discussions and to Drs. J. Amesz and L. M. N. Duysens for valuable advice. Financial support was given by the Netherlands Organization for the Advancement of Pure Research (ZWO) via the Foundations for Biophysics and for Chemical Research (SON).

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