Interactions of Protons with Transitions of the Watersplitting Enzyme of Photosystem II as Measured by Delayed Fluorescence

Jane M. Bowes * and Antony R. Crofts *

Department of Biochemistry, University of Bristol, Bristol, U.K.

Z. Naturforsch. **33 c**, 271–275 (1978) ; received February 13, 1978

Photosystem II, Watersplitting, Protons, Delayed Fluorescence

By measuring ms delayed fluorescence emission, we have investigated the interaction of protons in the reactions of the watersplitting enzyme of photosystem II.

(1) In the presence of the electron transport mediators 2,3,5,6-tetramethyl-p-phenylene diamine (DAD) (in its reduced form) and methyl viologen and of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), the maximal intensity of the delayed fluorescence measured between 1 and 1.5 ms after illumination was greater than the level reached in the presence of DCMU alone.

(2) The stimulation of the delayed fluorescence was greater when the suspension was preilluminated by 1–8 xenon (or laser) flashes prior to addition of DCMU, and the maximal intensity varied with the number of preilluminating flashes given.

(3) The amplitude of the intensity of delayed fluorescence oscillated with a periodicity of 4, with a maximum after 2, and minimum after 4 flashes. A negligible oscillation was observed in the absence of the electron transport mediators.

(4) When the prompt fluorescence intensity was measured under these conditions, a weak oscillation of period 4 with superimposed periodicity of 2 was observed in F_0, but of insufficient amplitude to account for the observed changes of delayed fluorescence intensity in terms of the back reaction caused by addition of DCMU.

(5) We therefore suggest that the enhancement of the delayed fluorescence is due to the release of protons accompanying transitions of the S-states, and that this release occurs not in synchrony with O_2, but in all the transitions of the S-states with the exception of $S_1 \rightarrow S_2$. We discuss the relative potentials of the transitions at the low internal pH which is generated as a result of cyclic electron transport around photosystem I in the conditions of our experiments.

Introduction

The release of protons in the photosynthetic watersplitting reaction has until recently been thought to occur as a concerted reaction, concomitant with oxygen evolution in the transition between S_4 and S_0 of the so-called S-states [1, 2]. More recent measurements of the yield of protons as a function of flash number, using pH sensitive indicator dyes [3, 4] or a glass electrode technique [5] have suggested that the release of protons does not occur simultaneously and stoichiometrically with that of oxygen, but that some protons are released in earlier transitions of the S-states. In this paper we present independent evidence obtained by measurement of the enhancement of the delayed fluorescence from photosystem II when the internal pH is lowered through activation of photosystem I, which supports the model of Saphon and Crofts [3] and of Fowler [5], for the release of protons in these transitions. In these experiments reduced diaminodurene (DAD) was used in the presence of the acceptor methyl viologen (MV) and of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to inhibit electron transfer from the primary acceptor of photosystem II, Q [6]. This pair of redox reagents provides a highly efficient coupled electron transport through photosystem I [7]. Wraight and Crofts [8] showed that in the presence of DCMU, delayed fluorescence from photosystem II was stimulated when a proton gradient was generated in this way, and suggested that the stimulation reflected the involvement of protons within the thylakoid in the equilibria of the watersplitting enzyme. Such a stimulation would not be anticipated in dark adapted chloroplasts limited to a single transition of the S-states by DCMU if proton release and oxygen evolution were simultaneous [2] as previously suggested, since no protonic equilibria were thought to occur in the early transitions ($S_0 \rightarrow S_3$) of the S-states. We have therefore measured the stimulation of delayed fluorescence under these conditions as a function of the number of the preilluminating flash before addition of DCMU, and we conclude

* Present Address: Department of Physiology and Biophysics, 524 Burrill Hall, University of Illinois, Urbana, Illinois 61801.

Requests for reprints should be sent to Ms. Jane M. Bowes, Department of Physiology and Biophysics, 524 Burrill Hall, University of Illinois, Urbana, Illinois 61801.

that the internal protons are in equilibrium with all transitions of the S-states, with the exception of $S_1 \rightarrow S_2$ [3, 5].

A brief preliminary report of this work has been published elsewhere [9].

Methods

Chloroplasts were prepared from greenhouse spinach by conventional procedures, using a medium containing 0.4 M sucrose, 0.05 M potassium phosphate buffer, pH 7.8 and 0.01 M NaCl [10], and stored in the dark on ice at a concentration of 2 – 3 mg/ml until use. For measurement of delayed fluorescence, samples were diluted in the same buffer to a chlorophyll concentration of 5 – 10 μg/ml. Before each experiment, samples were dark adapted for 10 min at room temperature.

Delayed fluorescence was measured between 1 and 1.5 ms with a rotating sector phosphoroscope as previously described [8]. Flash preillumination was provided by a xenon flash lamp (Wingent, Cambridge, U.K., 200 W snaked xenon flash tube to special design; 5 μs pulse width) equipped with a light guide, or by a Q-switched laser (Laser Associates, Slough, U.K., Model 252 YAG laser, frequency doubled to emit at 530 nm; pulse width 20 ns), both mounted at right angles to the rotating sectors. The phosphoroscope actinic light, (12 V, 60 W quartz halogen lamp) and preilluminating sources were filtered with a blue filter (Corning 9782) and the detecting photomultiplier (EMI 9659, extended S20 cathode) with a Wratten 70 filter. Fluorescence measurements could be made in the same apparatus by a photomultiplier shielded by a Balzers interference (688 nm) and Wratten 70 filter combination and mounted at right angles to the rotating sectors. For these measurements, the sector over the phosphoroscope actinic lamp was moved to the 'on' position, and fluorescence was measured in continuous light when the shutter was opened.

Unless otherwise indicated, the following regime was used in all experiments. Chloroplast were mixed with a medium containing 0.25 mM DAD, 0.1 mM methyl viologen and 1 mM ascorbate as indicated in the figure legends, in the dim light of the laboratory. The suspension was dark adapted for 10 min then subjected to 0 – 8 saturating xenon or laser flashes (1/640 ms). Approximately 0.5s after the last flash, 5 μM DCMU was added, and the delayed fluorescence or fluorescence was measured on excitation by actinic illumination 15s after this addition.

Results and Discussion

Dependence of delayed fluorescence on S-state

Fig. 1 shows the delayed fluorescence on illumination of dark adapted chloroplasts which had been preilluminated by 0 – 8 flashes prior to addition of DCMU. A separate trace shows the amplitude of delayed fluorescence on illumination of dark adapted chloroplasts under similar conditions but in the absence of the electron transport mediators (0 and 2 flashes of preillumination). It can be seen that the delayed fluorescence on illumination in the presence of DCMU was enhanced when reduced DAD plus methyl viologen were also present, as previously observed [8, 9]. A further stimulation was obtained when the suspension was preillumi-

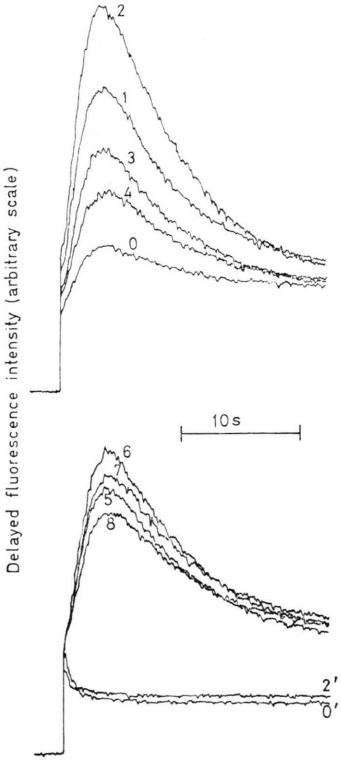

Fig. 1. The effect of preillumination on ms delayed fluorescence measured in conditions of lowered internal pH. The numbers indicate the number of preilluminating xenon flashes given (frequency 1/640 ms). Traces 0′ and 2′ were obtained in similar experiments in the absence of redox mediators for photosystem I. For details, see text.

nated by 1 – 8 xenon flashes prior to the addition of DCMU, and the maximal intensity of delayed fluorescence depended on the number of preilluminating flashes. A similar result was obtained using the Q-switched laser as a source of preilluminating flashes. In the preliminary report we were unable to observe this dependence (up to 3 flashes), but attribute this to the longer half pulse width of the flash lamp used for preillumination ($\cong 30\,\mu s$) [9].

The amplitude of the delayed fluorescence observed 5s after shutter opening has been plotted as a function of flash number in Fig. 2. The amplitude oscillates clearly with a marked periodicity of four, with maxima following preillumination by 2 or 6 flashes and minima after 0, 4 or 8 flashes. In separate experiments the time between the last flash and the addition of DCMU was varied between 0.5s (as in Fig. 1) and 20s, with no marked change in the pattern of dependence on preillumination. The time between addition of DCMU and the measuring illumination was also varied from 4s to 30s without any marked effect on the pattern of flash dependence. Although the overall pattern of flash dependence did not vary markedly with these timing regimes, similar experiments over a wider range of times, and with chloroplasts in which the state of the oxygen evolving apparatus was selected so as to favor S_0 or S_1 by appropriate flash preillumination and dark adaptation, could be used to follow the decay of the higher S-states in the presence or absence of DCMU. These results will be reported more fully in a later paper.

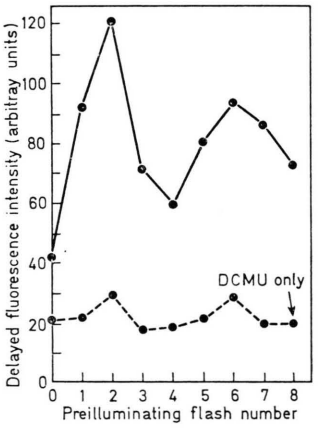

Fig. 2. Intensity of delayed fluorescence plotted as a function of flash number. The delayed fluorescence was measured under the conditions of Fig. 1. The lower trace shows the intensity in the absence of electron transport mediators.

Mechanism of enhancement

Photosystem II can be envisaged as spanning the thylakoid membrane, with the donor and acceptor sites on opposite sides and in equilibrium with secondary or tertiary pools [11], the midpoint potentials of which are dependent on the pH of the phase. The emission of delayed fluorescence occurs by reversal of the photochemistry, and its intensity is enhanced by a lowering of the activation energy required for the reaction as a result of the storage of energy in the redox state of the reaction center and in the electrochemical proton gradient [12].

While the electrical component of the proton gradient might be envisaged as directly lowering the activation energy of the back reaction, the pH gradient can only have its effect through the involvement of protons in the redox reactions of the donor and acceptor pools. On illumination of DCMU-inhibited chloroplasts in the presence of ascorbate, DAD and methyl viologen, photosystem I turns over and protons are pumped into the chloroplasts. The increased concentration of protons within the thylakoid would be expected to increase the oxidizing potential of reactants on the donor side by 60 mV/pH unit decrease for each proton involved in a single electron transition, but to have no effect on those reactions in which no proton was involved. Saphon and Crofts [3] and Fowler [5] have shown that protons are released within the thylakoid on all transitions of the S-states except that of $S_1 \rightarrow S_2$. The redox couples involved may therefore be represented as:

$$S_0 \rightarrow S_1 + H^+ + e\,, \tag{1}$$

$$S_1 \rightarrow S_2 + e\,, \tag{2}$$

$$S_2 \rightarrow S_3 + H^+ + e\,, \tag{3}$$

$$S_3 \rightarrow S_0 + O_2 + 2\,H^+ + e\,. \tag{4}$$

It can be seen that for each of the single electron transitions, (2) involves no proton, (1) and (3) involve one proton, and (4) involves two protons.

The dark distribution of S-states is found empirically to be 30% S_0 and 70% S_1 [1]. On continuous illumination in the presence of DCMU, photosystem II is able to turn over rapidly once only, so that the S-states undergo a single transition from $S_n \rightarrow S_{n+1}$ where n depends on the number of preilluminating flashes before addition of DCMU, and on the population of S-states in the dark adapted state. The yield of protons on continuous illumination in

the presence of DCMU would be expected to vary as a function of number of preilluminating flashes with a periodicity similar to that of the normal flash yield, but displaced by one flash. With the dark distribution of S-states as above, this would give a yield of 0.3, 0.7, 1.7, 1.3, etc., with 0, 1, 2 or 3, etc., preilluminating flashes respectively (ignoring double hits and misses).

The enhancement of delayed fluorescence observed in Fig. 2 shows a pattern of flash dependency in line with this periodicity. However it may be noted that at constant external pH, for each of the partial reactions described in equations (1) – (4) the change in internal pH would generate a change in oxidizing potential in the reaction (and hence a change in the redox energy conserved) proportional to the number (z) of protons involved;

$$E^{\circ}_{S_n} = E^{\circ}_{S_n} - z\left(2\cdot 303\,\frac{RT}{F}\right)\cdot \mathrm{pH} + 2\cdot 303\,\frac{RT}{F}\log_{10}\frac{(S_{n+1})}{(S_n)}\,.$$

The relative change in intensity of delayed fluorescence (L) attributable to this change in internal pH (pH^{int}) would be given by:

$$\frac{L_2}{L_1} = \exp\left[-z\cdot \Delta\,\mathrm{pH}^{\mathrm{int}}_{(2-1)}\right]$$

assuming the simple analysis suggested by Wraight and Crofts [8, 12]. The relatively small differences between the delayed fluorescence intensities attributable to the different S-states (Fig. 2) suggest that at the internal pH generated by PSI tunover (presumably about pH 4.5) [13] the different couples represented in equations (1) – (4) have similar redox potentials, ranged in order of decreasing potential as follows:

$$S_0\cdot 2\,H^+/S_3 > S_3\cdot H^+/S_2 \geqq S_1\cdot H^+/S_0 > S_2/S_1\,.$$

This order differs, as would be expected, from that at neutral pH, where the dark equilibrium and relative rates of relaxation suggest an order more like

$$S_3\cdot H^+/S_2 > S_2/S_1 > S_1\cdot H^+/S_0 \geqq S_0\cdot 2\,H^+/S_3\,.$$

Thus the reaction in which oxygen is evolved:

$$P^+ + S_3 \rightleftharpoons S_0 + 2\,H^+ + O_2 + P$$

which appears to go rapidly and spontaneously to the right at near neutral pH, seems to operate much closer to equilibrium at the internal pH (pH 4.5 – 5) likely to be present under conditions of maximal power conversion.

The state of acceptor side components

Interpretation of these results in terms of the simple model above takes no account of several possible complicating features. The most serious of these is the extent to which relaxation of the S-states could have occurred after addition of DCMU, due to a back reaction in which electrons from B^- [14 – 16] return to the S-states via Q and P [17, 18]. Velthuys [16] and Wollman [17] have attempted to measure this reaction by observing the dependence on the number of preilluminating flashes of several phenomena associated with fluorescence and delayed fluorescence. We have estimated the contributions of the reactions which affect the final level of Q, *i. e.*, the back reaction and transfer of charge stored as B^- to Q on addition of DCMU (see above), by measuring F_0, the instantaneous level of prompt fluorescence on il-

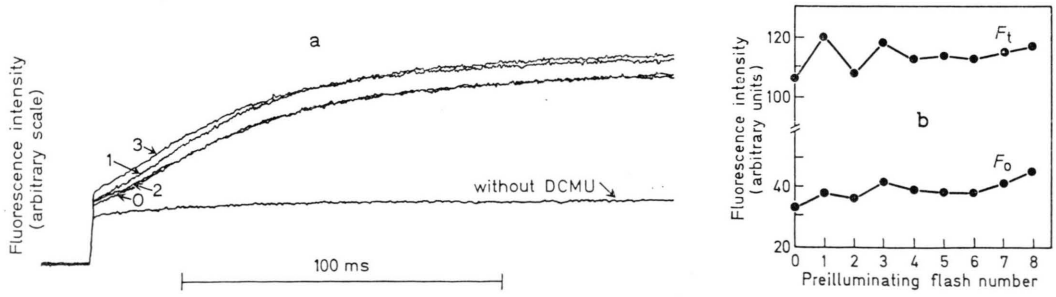

Fig. 3. Changes in fluorescence under the conditions of the delayed fluorescence measurements as a function of flash number. a) Traces are shown for 0–3 preilluminating flashes and for the fluorescence rise in the absence of DCMU (numbers indicate preilluminations). b) F_0 is the initial level of fluorescence, F_t is the fluorescence intensity at 178 ms after the onset of iliumination.

lumination, under the same conditions as the measurements of delayed fluorescence. We have been able to show an oscillation of period 4 with superimposed periodicity of 2 in the level of F_0, similar to that shown by Wollmann [17], but of lower amplitude (Fig. 3 a, 3 b). We were unable to observe any marked biphasic oscillation in the level of F_0 attributable to the acceptor side reactions in Tris-washed chloroplasts with phenylene diamine plus ascorbate or in normal chloroplasts with 10 mM hydroxylamine [15 – 17] under the conditions of our delayed fluorescence experiments. A consistent small biphasic oscillation in the level of fluorescence measured 178 ms after switching on the actinic light (F_t) was observed under our conditions, and this was reflected in the oscillations of F_{var}, but we could not account for the amplitude of the oscillations of delayed fluorescence in terms of the flash number dependency of the back reaction and dark state of B prior to DCMU addition. We therefore suggest that the enhancement is most satisfactorily explained as a consequence of proton release accompanying transitions of the S-states.

We are grateful to Dr. S. Saphon for stimulating discussions, to Mr. G. V. Tierney for development and maintenance of the electronic apparatus and for computer interfacing, and to Mr. E. Watson of Long Ashton Research Station, Bristol University, Bristol, U.K., for providing spinach. J. M. B. acknowledges the financial support provided by the Science Research Council of Great Britain. We are grateful to the Royal Society for a grant for development of the phosphoroscope.

[1] B. Kok, B. Forbush, and M. P. McGloin, Photochem. Photobiol. **11,** 457–475 (1970).
[2] C. F. Fowler and B. Kok, Biochim. Biophys. Acta **357,** 299–307 (1974).
[3] S. Saphon and A. R. Crofts, Z. Naturforsch. **32 c,** 617–626 (1977).
[4] W. Junge, R. Renger, and W. Ausländer, FEBS Lett. **79,** 155–159 (1977).
[5] C. F. Fowler, Biochim. Biophys. Acta **462,** 414–421 (1977).
[6] L. N. M. Duysens and H. E. Sweers, Microalgae and Photosynthetic Bacteria, Special Issue of Plant Cell Physiol., pp. 353–372, Tokyo Press, Tokyo 1963.
[7] G. A. Hauska, R. E. McCarty, and E. Racker, Biochim. Biophys. Acta **197,** 206–218 (1970).
[8] C. A. Wraight and A. R. Crofts, Eur. J. Biochem. **19,** 386–397 (1971).
[9] J. M. Bowes, S. Itoh, and A. R. Crofts, Abstracts of the Fourth International Congress on Photosynthesis, Reading, England, p. 50 (1977).
[10] S. Itoh, N. Murata, and A. Takamiya, Biochim. Biophys. Acta **245,** 109–120 (1977).
[11] P. Mitchell, Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation, Glynn Research Laboratories, Bodmin, Cornwall, England 1966.
[12] A. R. Crofts, C. A. Wraight, and D. E. Fleischmann, FEBS Lett. **15,** 89–100 (1971).
[13] B. Rumberg and U. Siggel, Naturwissenschaften **56,** 130–132 (1969).
[14] B. Bouges-Bocquet, Biochim. Biophys. Acta **314,** 250–256 (1973).
[15] B. R. Velthuys and J. Amesz, Biochim. Biophys. Acta **333,** 85–94 (1974).
[16] B. R. Velthuys, Thesis Rijksuniversiteit Leiden, The Netherlands 1976.
[17] F. A. Wollman, Thèse de Troisième Cycle, Paris 1977.
[18] A. L. Etienne and J. Lavorel, FEBS Lett. **57,** 276–279 (1975).