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A two-electron reaction at the catalytic site of water oxidation in manganese-depleted Photosystem II vesicles in the presence of an electron donor. Coupled electron and proton transfer processes

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Abstract

We have studied the electron and proton transfer at the manganese-depleted catalytic site of water oxidation in the presence of the electron donor 1,5-diphenylcarbazide (DPC). On the basis of measurements of flash-induced fluorescence yield, electrochromic bandshifts and pH transients into the aqueous medium, we have been led to the following conclusions: (1) The electron donor DPC was involved in a two sequential photostep process just like Mn^{2+} in photoactivation. After a first flash, the product of the reaction of DPC with the first Photosystem II charge separation was unstable (half-decay around 1 s at pH 6.5) until converted into a stable one by a second flash. The formation of the unstable intermediate was rather favored at alkaline pH. Its concentration increased progressively with pH to reach a maximum at pH 7.2–7.4. Nevertheless, consumption of this intermediate required a proton binding, since its regeneration after the second flash needed a latency dark time inversely proportional to proton concentration. (2) Each step of the process was accompanied by proton exchange with the medium. At acidic pH, the odd flashes in a series induced acidification, whereas on even flashes occurred alkalinization. The pattern of proton release/uptake varied extremely as a function of pH, between 4 H^+ and -2 H^+/e^-, with opposite changes at acidic and basic pH. At neutral pH, 1 H^+/e^- was assumed to be released at each step. On the basis of these results and data already in the literature, the molecular details of the pH-dependent part of the deprotonation are discussed in terms of electrostatic interactions between the polarized catalytic center and bound H^+ and OH^- ions. The described two-photostep process is proposed to reflect the two-electron water oxidation to an hydrogen peroxide intermediate.

Keywords: Photosystem II; Water oxidation; Photoactivation; Chlorophyll fluorescence; Electrochromism; Proton

1. Introduction

The water-oxidizing complex of PS II is a highly ordered structure in which a number of polypeptides interact with one another to provide the appropriate environment for efficient photooxidation of water to molecular oxygen (for reviews see Refs. [1,2]). The energy from a single photon generates a charge separation in PS II forming $P680^+$ (an oxidized chlorophyll) and $Pheo^-$ (a nearby reduced pheophytin). Stabilization of these species against charge recombination is achieved by the rapid (hundreds of ps [3]) oxidation of $Pheo^-$ by a nearby plastoquinone molecule Q_A and by the reduction of $P680^+$ by a redox-active tyrosine residue on the D_1 polypeptide termed Y_Z (within 300 ns [4,5]). Charge recombination between Q_A^- and Y_Z^+ is prevented by further electron transfer events: a second plastoquinone molecule, Q_B, oxidizes Q_A^- forming Q_B^-, and the water-oxidizing complex, which contains the manganese cluster, reduces Y_Z^+ within 30–1300 μs [6]. Under special conditions, such as after prolonged dark-adaptation or at low temperature, photooxidation of the Mn complex occurs in competition with photooxidation of a tyrosine residue on the D_2 polypeptide, Y_D, cytochrome *b*-559, and a chlorophyll molecule [2].

In accordance with the electrochemistry of water oxidation, the reaction center must undergo four one-electron

Abbreviations: Chl *a*, chlorophyll *a*; PS, Photosystem; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, 2, amino-2-hydroxymethylpropane-1,3-diol; BCP, bromocresol purple; DPC, 1,5-diphenylcarbazide; PBQ, phenyl-*p*-benzoquinone.

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photoreactions to produce the four oxidizing equivalents required for the generation of an oxygen molecule [7]. The redox states of the cluster with 0–4 positive equivalents are generally called the S_0–S_4 states respectively. On the acceptor side, after two turnovers of PS II, plastoquinol (Q_BH_2) is generated and can exchange with an oxidized molecule of the plastoquinone pool with the return to the initial oxidized state of the acceptor quinone complex (Q_AQ_B) [8].

In untreated PS II membranes, the flash-induced fluorescence yield has been observed to oscillate with a periodicity of four. This is consistent with the fluorescence yield being susceptible to events on the donor side, such as the presence of a net excess of positive charge in the S_2 state [9].

The Mn cluster is destroyed by treatment with Tris and is accompanied by the release of PS II extrinsic proteins and a loss of O_2 evolution activity. O_2 evolution activity and the Mn complex can be reconstituted in PS II through photoactivation, which is a process characterized by light-driven incorporation of Mn atoms into the depleted catalytic center. This process requires at least two sequential photochemical events [10]. This has been demonstrated with various cultured algae [10,11], chloroplasts [12] and PS II membranes depleted of Mn by various means [13,14]. The primary step of photoactivation is considered to be Mn^{2+} photooxidation, i.e., Mn^{2+} acting as an electron donor [13,15]. The electron donation reactions during the primary step of photoactivation were found to be identical with those in untreated PS II, except that electron donation from the Mn complex was absent and could be replaced by slower electron donation from exogenous Mn^{2+} [16]. In particular, photooxidation occurs in competition with photooxidation of the tyrosine residue Y_D, and cytochrome *b*-559, Thus, these two species are excluded from direct participation in the assembly of the Mn complex [16]. In contrast, Mn^{2+} is thought to be oxidized by Y_Z^+ since it can serve as an indicator of photooxidation of Y_Z [17].

In order to obtain an insight into the structure and the binding sites of the Mn-depleted catalytic complex, we have studied the flash-induced reactions in Mn-depleted PS II membranes in the presence of DPC as an electron donor. We have measured flash-induced fluorescence yield, electrochromic bandshifts as well as pH transients into the aqueous medium as a function of pH. The experiments discussed in this paper were designed to identify the similar reactions that occur whatever the kind of electron donor in Mn-depleted PS II membranes. We have obtained new information about a two-photostep process that was initially studied during photoactivation. Our results provide direct evidence that each step of this process is also associated with pH-independent and pH-dependent proton exchange with the aqueous phase. The reaction that proceeds as a two-electron process is suggested to be the two-electron water oxidation to an hydrogen peroxide intermediate.

2. Material and methods

Inside-out thylakoids were obtained by mechanical disintegration of pea chloroplasts thylakoids, followed by phase partitioning according to Ref. [18]. Tris-treated inside-out thylakoids were prepared by incubation with 0.8 M Tris-HCl (pH 8.0) for 30 min in darkness. The treated membranes were washed twice and then suspended in 300 mM sorbitol, 10 mM NaCl, 40 mM Mes-NaCl (pH 6.5). These preparations were used directly. The following buffers were also used: bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis-Tris), $6.0 < pH < 7.2$; *N*-tris(hydroxymethyl)methylglycine (Tricine), $7.4 < pH < 8.0$.

Flash excitation was provided by Stoboslave General Radio flashlamps (3 μs at half-peak height).

Fluorescence experiments were performed using the apparatus already described [19–21]. The fluorescence after darkness F_0, was subtracted from the fluorescence yield after each flash, F.

Optical changes were measured with a locally designed instrument as described previously [9] with slight modifications. The signal just before each flash of a series (1/10 of the full memory) and the absorption change up to 180 ms (9/10 of the full memory) were registered. Electrochromic absorption changes were measured at 515 nm.

Proton release was monitored optically using the pH indicator dye Bromocresol purple (BCP) [22] at a concentration of 7 μM. Inside-out thylakoids in the presence of BCP were diluted at 10 μg of Chl/ml in a 10 mM NaCl solution. Firstly, the absorption changes of BCP at 580 nm were measured for an inside-out thylakoid suspension without buffer. The repetitive signals were summed in an IBM-compatible computer and then the same number of signals was subtracted, obtained from the same suspension in the presence of buffer (40 mM). The plus-buffer control was used to correct for non-bufferable background responses [23,24]. Prior to and after each flash series, the medium pH was measured and adjusted if necessary. The change in absorption was calibrated by adding known amounts of HCl.

3. Results

3.1. Fluorescence yield as a function of flash number

Evidence for a two-photostep process. In Tris-washed PS II membranes in the presence of 0.5 mM ferricyanide, the flash-induced fluorescence yield measured 80 ms after each flash of a series, almost immediately reached a constant level as a function of flash number after a thorough (20 min) or a 6-min dark-adaptation period (Fig. 1a,b). The relatively low fluorescence yield after each flash, slightly higher than found in untreated PS II membranes, indicates that Q_A^- was almost reoxidized as in

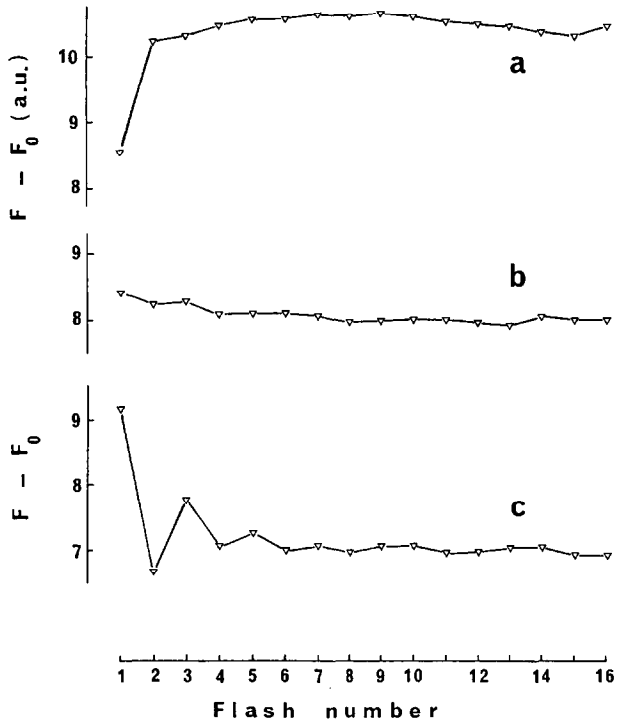


Fig. 1. Flash number-dependent fluorescence yield measured 80 ms after each flash, in the absence (a,b) and presence of DPC (c), in Tris-treated inside-out thylakoids at pH 6.5 in the presence of 0.5 mM ferricyanide. (a) no DPC, no further addition, the dark adaptation period was 1 h. (b) no DPC, 0.25 μg/ml gramicidin, the dark adaptation period was 6 min. (c) 200 μM DPC, 0.25 μg/ml gramicidin, 20 μM PBQ. The spacing between flashes was 1.3 s.

untreated PS II membranes. The addition of the reductant DPC induced large binary oscillations of fluorescence yield as a function of flash number. The ability to produce binary oscillations was not changed when 20 μM phenyl-*p*-benzoquinone (PBQ) was added to accept electrons, as shown in Fig. 1c. Thus, we attempted to get an insight on how electron donation reactions in Mn-depleted PS II could induce these changes.

Fig. 2 (left panel) shows the dependence on flash interval of the flash-induced pattern of fluorescence yield in the presence of DPC. At short interval ($\Delta t = 360$ ms), the fluorescence yield after the second flash (F_2) was depressed significantly in comparison with the fluorescence yield after the first flash (F_1). With increase of the flash interval, the observed depression equal to $F_1 - F_2$ diminished (see also Fig. 3, curve 1). When the flashes were spaced too far apart (> 9 s), the conversion of a component responsible for the high fluorescence state (F_1) to a second component of lower fluorescence state (F_2) did not occur. To account for the observed kinetics, we need to assume minimally the involvement of two sequential photosteps, and that one photochemically formed intermediate has limited stability since the effect of the first quantum event rapidly disappears. Interestingly, the shape of curve 1 in Fig. 3 (half-decay of about 1 s) agrees with previously reported findings on the yield of photoactivation, i.e., the yield of restoration of O_2 evolution activity when the Mn-containing catalyst is assembled from Mn^{2+} [13].

The effect of ionic screening on the Mn-depleted PS II

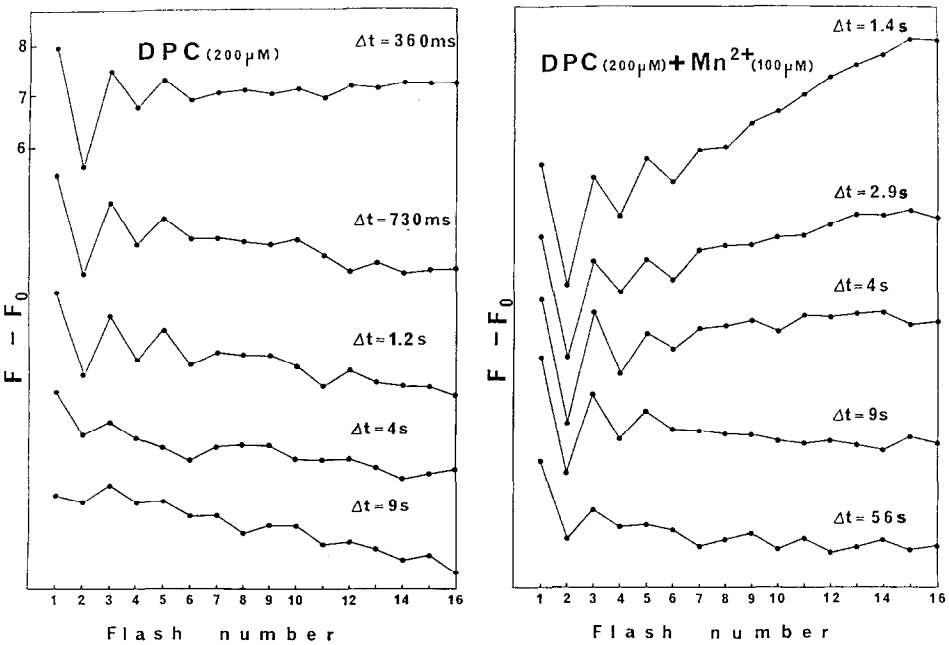


Fig. 2. Oscillation pattern of fluorescence yield as a function of flash interval, in Tris-treated inside-out thylakoids at pH 6.5 in the presence of 200 μM DPC (left panel), and in the presence of 200 μM DPC + 100 μM $MnCl_2$ (right panel). Other additions: 0.5 mM ferricyanide, 0.25 μg/ml gramicidin. The dark adaptation period was 6 min. For all of the sequences, the level of fluorescence yield after the first flash was the same; the curves have been offset for presentation.

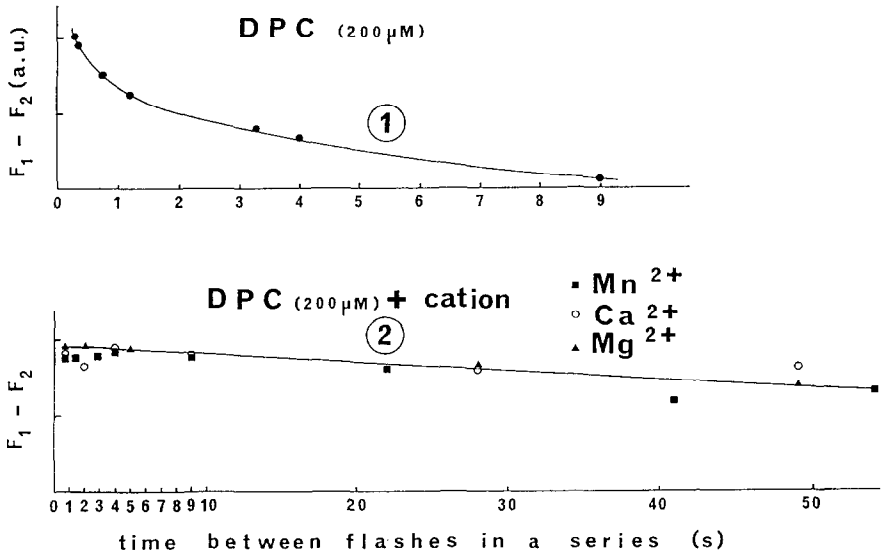


Fig. 3. Flash-interval dependence of the difference between the fluorescence yield after the first flash and the fluorescence yield after the second flash, $F_1 - F_2$, in Tris-treated inside-out thylakoids at pH 6.5 in the presence of 200 μM DPC (curve 1) and in the presence of 200 μM DPC + 100 μM divalent cation, either Mn^{2+} ($MnCl_2$), Mg^{2+} ($MgCl_2$) or Ca^{2+} ($CaSO_4$). Same conditions as in Fig. 2.

was to stabilize the two-photostep process. The addition of $MnCl_2$, $MgCl_2$ or $CaSO_4$ (50 μM–100 μM) made favorable the formation of large successive binary oscillations as a function of flash number, even with a dark interval between flashes as long as 22 s (Fig. 2, right panel). The presence of divalent cations, by decreasing the interaction energy between the charges in the enzyme, seems to suppress the decay of the unstable intermediate as shown in Fig. 3, curve 2. However, the increase in the ionic strength slowed the rate of Q_A^- reoxidation considerably. As shown in Fig. 2 (right panel), the flash-induced fluorescence yield progressively increased as a function of flash number at flash interval < 9 s. This indicates that a fractional amount of Q_A^- did not undergo reoxidation to Q_A prior to the application of the following actinic flash, increasing the proportion of photochemically inactive reaction centers. The addition of 20 μM PBQ accelerated the electron transfer under these conditions, by reducing the progressive increase of the fluorescence yield at flash interval < 9 s (not shown). Submicromolar concentrations of the divalent cation Mn^{2+} have been reported to inhibit electron donation by DPC to Mn-depleted PS II mem-

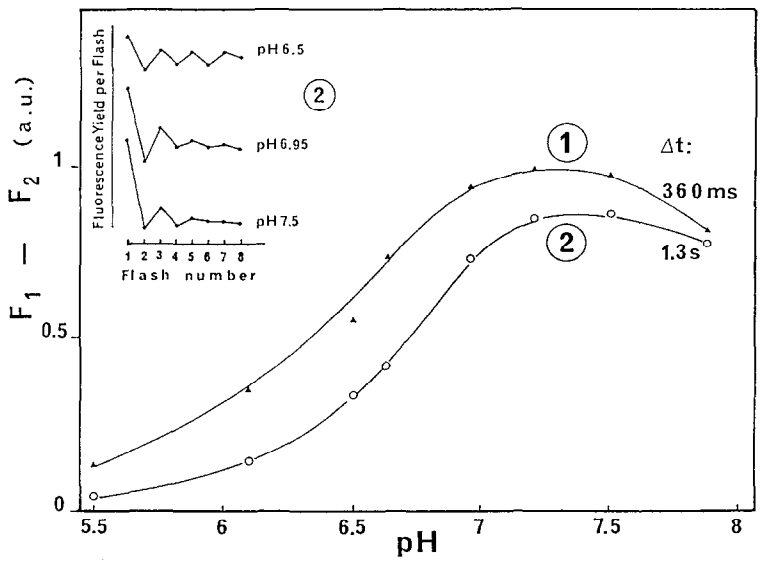


Fig. 4. pH dependence of the difference between the fluorescence yield after the first flash and the fluorescence yield after the second flash, $F_1 - F_2$, at two different flash-intervals: 360 ms (1) and 1.3 s (2), in Tris-treated inside-out thylakoids in the presence of 200 μM DPC. Inset: oscillation patterns of fluorescence yield obtained at pH 6.5, pH 6.95 and pH 7.5, at a flash interval of 1.3 s. Other additions: 0.5 mM ferricyanide, 0.25 μg/ml gramicidin. The dark adaptation period was 6 min.

branes [25,26], in a non-competitive interaction [26] (as measured by the photoreduction of 2,6-dichlorophenol-indophenol). We do not have sufficient evidence that this result is identical with ours because of the differences in experimental conditions.

pH dependence of the two-photostep process. Fig. 4 shows a relatively important pH effect on the proportion of PS II centers able to undergo a two-photostep process on the second flash. The inset of Fig. 4 displays an example of the binary oscillations obtained at different pH (the flash spacing was 1.3 s). In the presence of DPC, the depression of the fluorescence yield on the second flash, $F_1 - F_2$, was small below pH 5.5, progressively increased with pH to reach a maximum at pH 7.2–7.4, which was then followed by a gradual decrease at higher pHs. The curve shown in Fig. 4 closely resembles the pH dependence of the thermoluminescence band, termed the A_T band, with peak intensity at around -20°C observed in Tris-treated PS II membranes [27]. The A_T band has been suggested to arise from charge recombination between an oxidized species on the donor side and Q_A^- [27]. This could indicate that the same protonation event controlled the yield of the species responsible for the thermoluminescence A_T band and the yield of the stable component formed on the second flash in the presence of DPC. It was also shown that in Tris-treated PS II membranes, protolytic reations are involved at the level of Y_Z, in particular the rate of the electron transfer between Y_Z and $P680^+$ slows down as the pH decreases [28].

Recovery of the two-photostep process. By comparing the fluorescence yield after the third flash (F_3) to the fluorescence yield after the second flash (F_2), we studied the conditions for a maximum recovery of the intermediate. Fig. 5A and the inset of Fig. 4 describe the progressive effect of increasing pH values on the oscillation pattern of fluorescence yield obtained with different flash-spacings. When the flash-spacing was 360 ms, the difference $F_3 - F_2$, considerably decreased with increasing pH. At pH 7.5, no binary oscillation was noticeable after the first pair of flashes. This result reveals the necessity of a waiting time preceding each pair of flashes at pH > 7, since significant binary oscillations were observed at pH 7.5, for longer flash spacings, 2.9 s as example (Fig. 5A). These data indicate that the consumption of the intermediate requires a proton binding, since the dark time for a second binary oscillation to occur, increased with increasing pH. Thus,

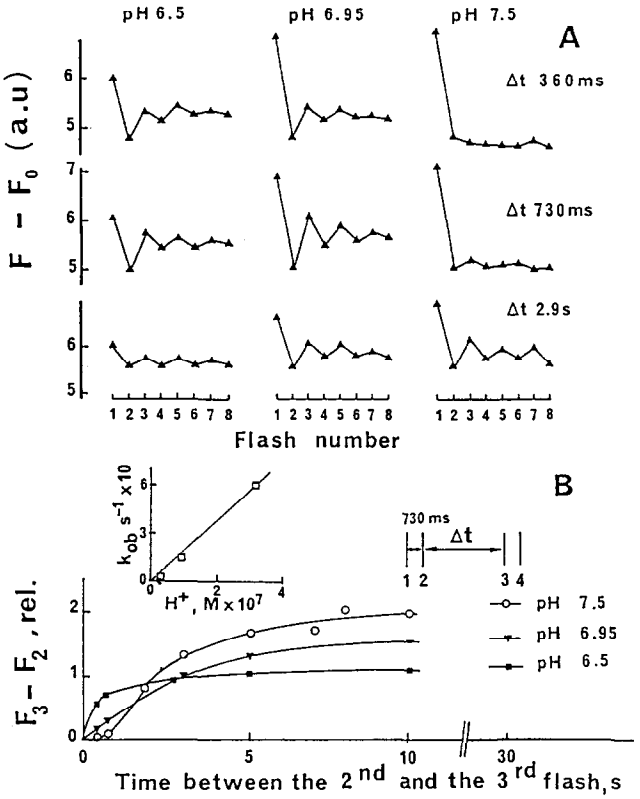


Fig. 5. Effect of pH and flash-interval on the fluorescence yield after the third flash in a series, F_3, in Tris-treated inside-out thylakoids in the presence of 200 μM DPC. (A) pH (columns) and flash interval (lines) dependence of the oscillation pattern of fluorescence yield. (B) Recovery of the initial fluorescence yield on the third flash, $F_3 - F_2$, as a function of the time interval between the second and the third flash at pH 6.5, pH 6.95 and pH 7.5. Other flashes were spaced 730 ms apart. The maximum recovery was obtained for a time interval of about 30 s. In panel B inset, the apparent first-order rate constants calculated from the first phase of the recovery kinetics were plotted as a function of proton concentration in the medium. Other conditions were as in Fig. 4.

we studied the recovery kinetics of the high fluorescence yield after the third flash, by keeping constant the flash interval between the first and the second flash (730 ms), and by varying the dark time between the second and the third flash in a series. As shown in Fig. 5B, the recovery kinetics was markedly biphasic. The apparent first-order rate constants calculated from the first phase were found to be proportional to $[H^+]$ in the medium (Fig. 5B, inset). Thus, the kinetic first phase appears to be related to one proton binding, prerequisite condition to the completion of the second phase.

3.2. Absorption changes at 515 nm

The flash-induced absorption changes at 515 nm, attributed to electrochromic bandshifts of carotenoids, have been shown to reflect the transmembrane electric field, as well as the local influence of a surplus of charge on the donor side [9,29]. In untreated PS II membranes, the oscillation pattern of this signal appears to be similar to the period four oscillation of the chlorophyll *a* bandshift [30].

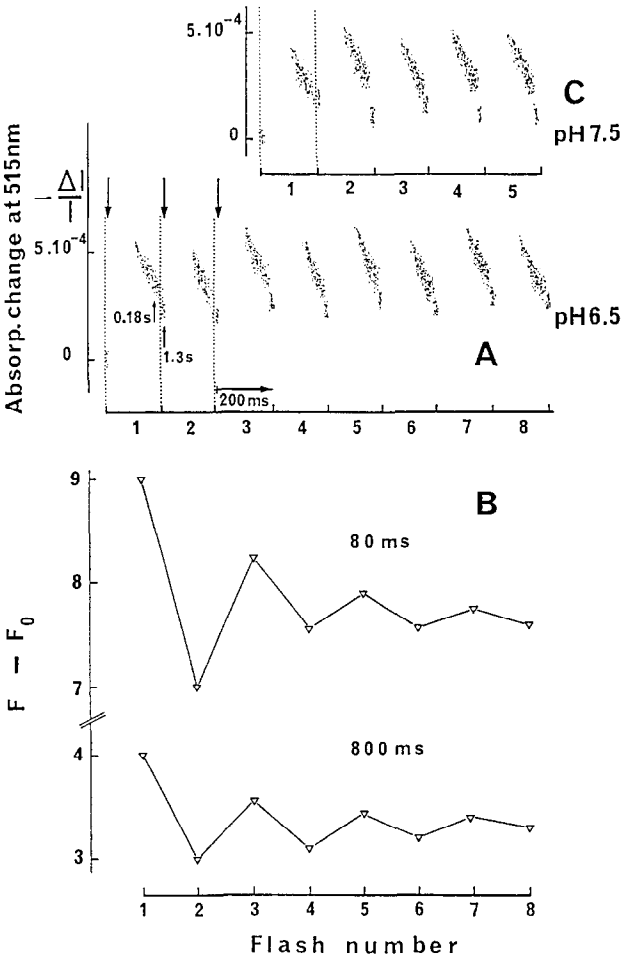


Fig. 6. In dark-adapted Tris-treated inside-out thylakoids, flash-induced absorption changes at 515 nm from around 70 ms to 180 ms after each flash and every 1.3 s (the signal just before the flash was registered) (A), in comparison to fluorescence yield measured at 80 ms and at 800 ms (B), at pH 6.5 in the presence of 200 μM DPC, 0.25 μg/ml gramicidin, 5 μM $MgCl_2$ and 0.5 mM ferricyanide. The time per sweep was 200 ms, the spacing between flashes was 1.3 s, the dark adaptation period 6 min. (C): Flash-induced absorption changes at pH 7.5 under similar conditions. Arrows indicate the position of flash excitation. For absorption changes, 20 measurements were averaged.

Fig. 6A shows that at pH 6.5, in the presence of gramicidin in order to reduce the transmembrane electric field, the pattern of absorption changes at 515 nm oscillated with a periodicity of two. We observed increased absorption changes on odd flashes and decreased absorption changes on even flashes in the presence of DPC. Electrochromic absorption change at 515 nm and fluorescence yield appear to be correlated in time at pH 6.5, comparing Fig. 6A and B. It has been reported that upon electron transfer from Q_A^- to Q_B, electrochromic bandshifts due to interaction of Q_A^- with pheophytin *a* disappeared completely in less than 10 ms [31,32]. Thus, the electrochromic absorption changes observed some hundreds ms after each flash of a series, are not expected to be induced by acceptor side contributions. We suggest that the observed field-related changes originate from a charge excess of DPC when photooxidized in the Mn-depleted catalytic center. The observed binary oscillations support the view that DPC could undergo successive oxidation-reduction cycles under flashing light. Fig. 6C shows that at pH 7.5 the pattern of absorption changes at 515 nm appears to oscillate in opposite phase to the pattern of fluorescence yield (in Fig. 5A), when measured at time < 180 ms after each flash of a series. This result reflects the fact that electrochromic bandshifts and fluorescence yield variations may be considered as two different probes for electric field changes. The electrochromic probe responds to the net charge resulting from electron and proton movements. In contrast, the fluorescence yield seems solely susceptible to a change in electric field in the vicinity of the reaction center [9].

3.3. Protolytic reactions at the donor side

The protonation equilibrium in the PS II complex is under the influence of the redox states of components on the donor side, and of the acceptor quinones, Q_A/Q_A^-, Q_B/Q_B^-. Protons at the acceptor quinone site are equally taken up from the medium after each flash of a series [33]. In inside-out thylakoids, which are sealed vesicles with the inner thylakoid surface turned outside [18], the protons for the reduction of quinones are taken from the internal space in principle. Thus, by detecting pH changes in the outer aqueous phase, we could obtain transients essentially due to protolytic reactions at the donor side.

Fig. 7A shows transient absorption changes of the bromocresol purple (BCP) which are indicative of pH transients in the suspending medium. The downward-directed transient reflects the acidification caused by proton release at the Mn-depleted catalytic center in the presence of DPC. At pH 6.5, between 50 ms and 1.3 s after each

flash of a series, a net acidification was induced by odd flashes, whereas after even flashes occurred alkalinization. The observed proton transients appear to be related to the two sequential photosteps observed in the fluorescence oscillations. Firstly, a small amount of divalent cations (5–10 μM $MgCl_2$) stabilized the pH transients of opposite direction when the flashes were spaced 1.3 s (this amount was added in the suspension). Secondly, in the absence of divalent cations, the increase of the flash-spacing to 10 s resulted in the complete disappearance of these stabilized pH variations, as was observed for the period 2 fluorescence oscillations. Acidification followed by alkalinization occurred prior to the next flash in a series, so that no pH variations were noticeable 10 s after each flash.

The extents of the successive proton release and uptake at pH 6.5, determined 1.3 s after each flash, were plotted as a function of flash number (Fig. 7B, left panel). We found that the release after the first and the third flash was roughly around 1 proton per 600 Chl and the uptake after the second flash 2 times smaller. The pattern of flash-induced proton transients was measured at a medium pH ranging from pH 5.8 to 7.4. Fig. 8 shows the pH dependence of the respective extents of the first three flashes in a series. These extents depend on the sensitivity of the indicator (Fig. 7C), and the H^+ release reference. Usually, H^+ release stoichiometry is expressed in terms of the active reaction center content. This total content cannot be estimated, since in the two-photostep process, the first photochemically formed component has limited stability which depends on flash interval and pH. However, we have obtained by fluorescence measurements, in Fig. 4, the relative fraction of PS II centers competent to undergo the two-photostep process as a function of pH. This has enabled us to determine the relative stoichiometry of H^+ release/uptake associated with the active centers. The result is shown in Fig. 8, lower panel, where the extent of proton release after the first and the third flash and the extent of proton uptake after the second flash appear to be relatively constant when going from pH 5.8 to 6.5. Between pH 7.1 and 7.4, the opposite behavior was observed, i.e., proton uptake after odd flashes and proton release after even flashes. The crossing point where few protons were released after each flash was at pH 7.1. For unknown reasons, the extent of proton release/uptake after the first

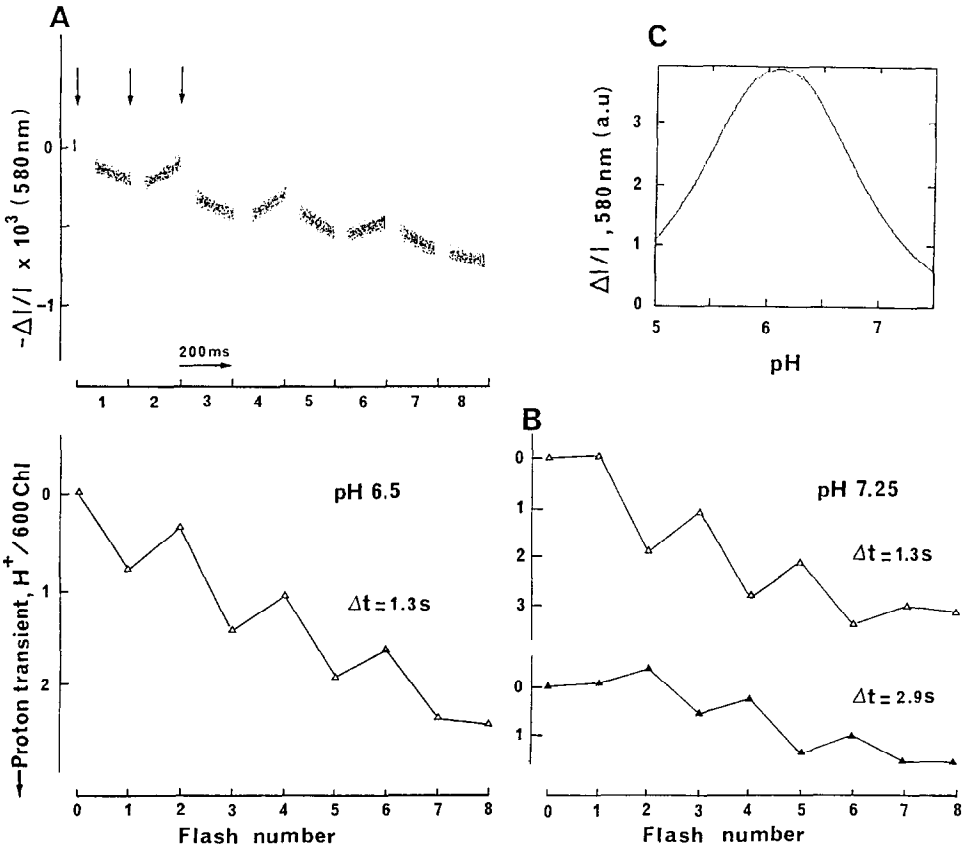


Fig. 7. pH transients in the external medium induced by a series of flashes in dark-adapted Tris-treated inside-out thylakoids in the presence of 200 μM DPC. (A): Flash-induced absorption changes of bromocresol purple (BCP) at 580 nm; 10 μg Chl/ml in 10 mM NaCl, 0.25 μg/ml gramicidin, 5 μM $MgCl_2$, 0.5 mM ferricyanide, 7 μM BCP and pH 6.5; the time per sweep was 200 ms, the flashes were spaced 1.3 s apart, the dark adaptation period was 10 min and 10 measurements were averaged. (B): Proton transients as a function of flash number at pH 6.5 (left panel) and pH 7.25 (right panel). At pH 7.25, the flash spacing was either 1.3 s or 2.9 s. The downward-directed transients reflect acidification. (C): Calibration curve of the proton release scale obtained by measuring the absorption change of BCP in response to an equal amount of protons to the suspension.

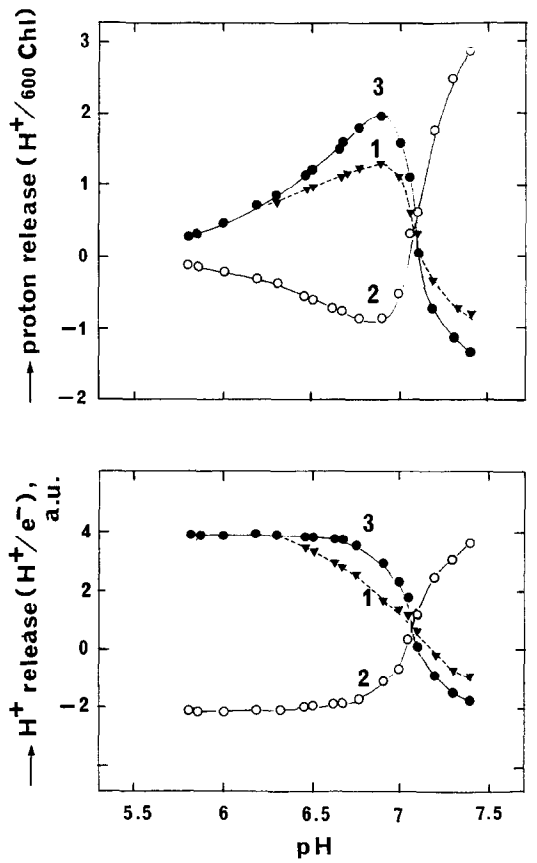


Fig. 8. Extent of pH changes induced by the first (1), the second (2) and the third (3) flash in a series of flashes spaced 1.3 s apart, as a function of pH in dark-adapted Tris-treated inside-out thylakoids in the presence of 200 μM DPC. Other conditions were as in Fig. 7. Each point is the average of four determinations. Upper panel: pH dependence of proton release/uptake per 600 chlorophylls. Lower panel: same per active center in arbitrary units.

flash was less than the extent of proton release/uptake after the third flash. The smaller proton variation after the first flash is presumably associated with the state of the reaction centers after a dark adaptation period (6–10 min) or with the presence of FeCy.

Fig. 7B shows the effect of a flash interval longer than 1.3 s (2.9 s) on the extents of proton release/uptake at pH 7.25. At this pH, with a flash interval of 1.3 s, the observed proton variations after the odd and the even flashes were reversed in comparison to those at pH 6.5. The extension of the flash interval to 2.9 s induced a resynchronization of proton release on odd flashes and proton uptake on even flashes. This could be explained by a partial reversion to the initial state after each flash.

4. Discussion

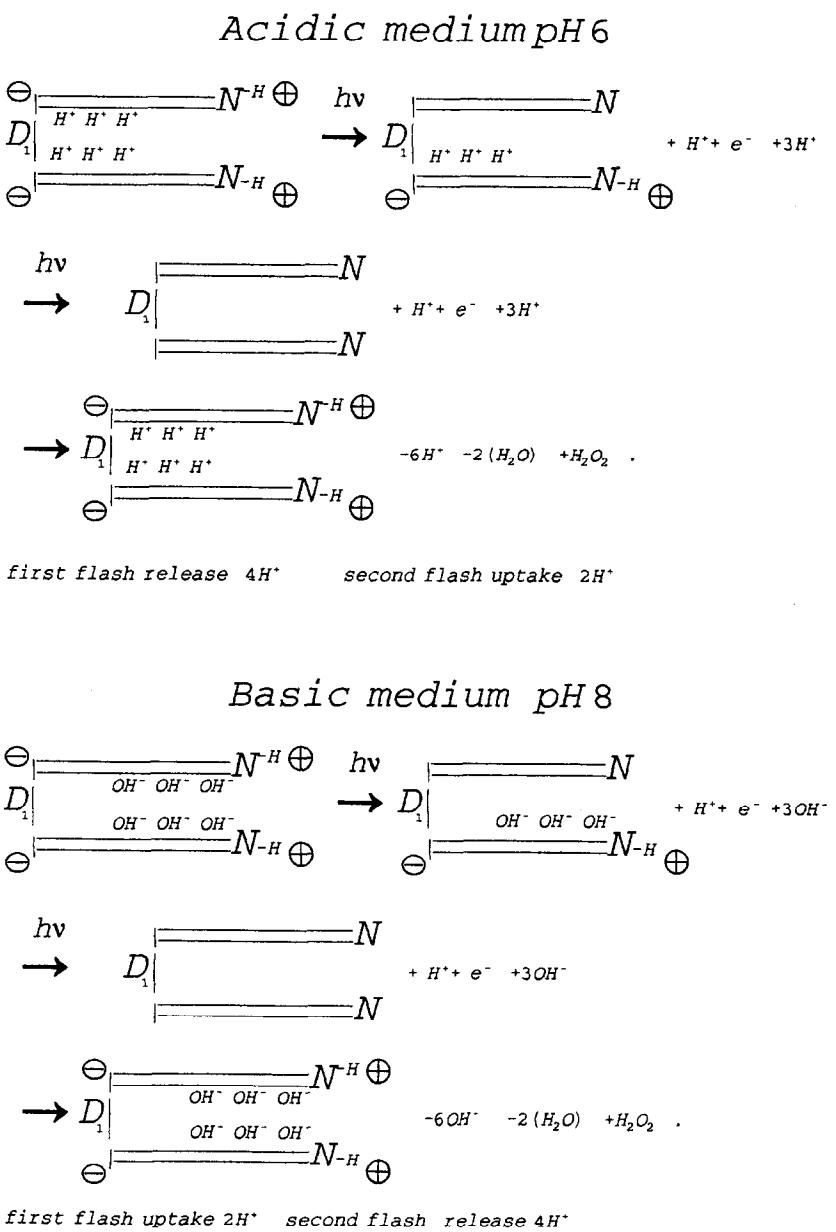
We have used flash-induced fluorescence yield, electrochromic bandshifts and proton release measurements to study the electron and proton transfer reactions in Mn-depleted PS II membranes in the presence of an electron donor (DPC). The observed fluorescence kinetics can be interpreted to reflect a series mechanism in which the product of the reaction of DPC with the first PS II charge separation is unstable until processed by a second charge separation. Almost the same conclusion was reached [34] when in photoactivation exogenous Mn^{2+} acts as an electron donor. The mechanism of photoactivation involves two light-dependent steps, and two photochemical events suffice to produce a stable intermediate that completes the assembly of the catalytic Mn complex of PS II, for the restoration of the O_2 evolution activity [34]. The half-decay time of the unstable intermediate observed in the presence of DPC, around 1 s, agrees fairly well with the value reported in photoactivation [13]. Furthermore, we roughly obtained the same results as described in this paper with Mn^{2+} (20 μM) instead of DPC as the electron donor. The similarity of the reactions when DPC instead of Mn^{2+} is the electron donor indicates that the function of the two-photostep process is not exclusively the incorporation of Mn into PS II and the assembly of the Mn cluster.

Our results imply that at the first step, the presence of DPC results in the formation of a positively charged oxidizing equivalent in the vicinity of P680 (oxidized DPC), responsible for the significantly enhanced amount of $P680^+$ Q_A^-, whereas at the second photostep, the charged equivalent is reduced. In the absence of an exogenous electron donor, the same event as the first step of this process seems to occur at low temperature, since Ono and Inoue have suggested that the thermoluminescence A_T band peaking at around -20°C arises from an oxidized species on the donor side [27]. The presence of an exogenous electron donor appears to increase significantly the stability of an unstable intermediate, that may complete the two-photostep process at the following flash instead of back-reacting. Our results indicate that the formation of the intermediate is rather favored at basic pH, with a maximum yield at pH 7.3–7.4. However, under conditions of successive two-photostep processes, we have shown that proton binding is required at the end of the second photostep. The regeneration of the relatively stabilized intermediate was found to depend on a latency dark time inversely proportional to proton concentration. So, our fluorescence results reveal that the steps of formation and consumption of the intermediate require opposite trends in pH conditions (see also Ref. [35]). This information provides new insight into the interpretation of the damping of the binary oscillations of fluorescence yield.

Our results also make evident that the two-photostep process is tightly associated with proton exchanges with the aqueous phase. In Tris-treated PS II membranes, in the absence of exogenous electron donor, proton release and uptake have been shown to be linked to the redox reaction of the electron carrier Y_Z [22,36,28]. The half-time of 140 ms found for the rebinding of proton to the releasing site is of the order of the reduction time of Y_Z^+ [17,28]. After

addition of DPC, the observed event was different. At acidic pH, provided that the flash interval was short or small amounts of cation present, proton ejection after odd flashes was coupled with the formation of an intermediate, stable enough to act as source of H^+ release exclusively, whereas proton uptake occurred after even flashes. In the presence of DPC, the lifetime of Y_Z^+ becomes about a few hundred μs [17], since DPC reduces directly Y_Z^+ [37]. Thus, proton release and uptake were mainly associated with the redox reaction of DPC, instead of Y_Z.

Proton release/uptake at the catalytic site of water oxidation may have different origins [38]: (1) chemical production of protons upon oxidation of water; (2) chemical deprotonation of ligands to the manganese cluster (when it is present); (3) deprotonation of surrounding aminoacids as an electrostatic response to the modification in charge distribution in the center after the flash. The curves of extent of proton release/uptake after 1 (or 3) flash(es) (oxidized DPC) and 2 flashes (reduced DPC) were strongly pH-dependent (Fig. 8). pH-dependent proton release has been reported by Haumann and Junge [39] in O_2-evolving pea thylakoids. The respective proton release for the transitions $S_1 \rightarrow S_2$ and $S_3 \rightarrow S_0$ has been found to exhibit a mutually compensating behavior that resembles



Scheme 1. Proposed mechanism for explaining the pH dependent proton release/uptake. Geometric symbol: polypeptide D_1. At the surface of the Mn-depleted center, the polarization charges attract the medium molecules and ions like H^+ and OH^- by electrostatic interactions. After the photon excitation that induces the release of a proton from an aminoacid (histidine), the polarization is smaller than before, leading to a smaller attraction of the ions. The attracted ions are released.

the pH dependence curves of this paper. Proton release variation with pH in O_2-evolving thylakoids has been explained by electrostatically induced shifts of acid/base equilibria of peripheral amino acids. In these experiments, the pattern of proton release was in the ratio 0.5, 1, 1.5, 1 for the first 4 flashes at basic pH, and 1.5, 1, 0.5, 1 at acidic pH (starting from S_1 to S_0). The mean value over all pH was 1, 1, 1, 1. These results indicate that the electrostatic effects stored 0.5 protons on $S_1 \rightarrow S_2$ and released them on $S_3 \rightarrow S_0$ at basic pH and that the reverse event occurred at acidic pH.

A simple model that incorporates these results is the possibility of large variations of the protein polarization at the catalytic site, as a function of flash number. For example in the starting state, the catalytic site is polarized with an equal number of negative and positive charges that attract positive H^+, negative OH^- ions and water molecules. At acidic pH, more H^+ are attracted to the protein, and at basic pH more OH^- ions are attracted. After a flash, the catalytic center becomes less polarized, leading to the release of the attracted ions. The released H^+ and OH^- ions are then added to the chemically produced proton release with opposite changes at basic and acidic pH. According to this model and the results reported by Haumann and Junge [39], the S_1 and the S_0 states are more polarized than S_2 and S_3 because the pH effect is maximum on the first and the third flash. The S_2 and S_3 states possess the same polarization since no pH effect was observed on the second flash, from S_2 to S_3. Approx. 0.5 H^+ were attracted to the catalytic site at acidic pH and 0.5 OH^- ions at basic pH [39].

The most likely reaction path for water oxidation is the two-electron water oxidation to hydrogen peroxide with the chemical release of two protons [40,41]. On account of this, the pattern of proton release/uptake associated with the two-photostep process was analysed under the assumption that the first two flashes in a series released two protons. Therefore, in Mn-depleted PS II vesicles in the presence of DPC, the released protons were found in the ratio 4, −2 at acidic pH, and −2, 4 at basic pH, as shown in Fig. 8. The mean value between acidic and basic pH: 1,1 corresponds to the pattern of the chemically released protons. A mechanism explaining the drastic pH-dependent proton release/uptake is shown in Scheme 1. In the dark, 3 H^+ are attracted to the negative part of the catalytic center at acidic pH, and 3 OH^- are attracted to the positive part of the polarized center at basic pH. After the first flash, the center is quite less polarized and 3 H^+ ions (or OH^- ions) are released. The reverse event occurs after the second flash when the center becomes again polarized. These ions are released independently of the chemically produced protons. This interpretation is supported by the observed stoichiometry: $4 = 1 + 3$ and $-2 = 1-3$ protons at acidic pH and the reverse ratio at basic pH. Scheme 1 predicts that the polarization of the catalytic center was different at acidic and basic pH with opposite electric fields, due to the presence of attracted ions of opposite signs, offering an explanation to the opposite variations of the absorption changes at 515 nm as observed. Scheme 1 also implies that at any pH, the release of the two-chemical protons, one after each step, is necessarilly followed by the rebinding of these protons for the restoration of the polarized center. Being located on different sites, each proton is expected to be taken up with a rate proportional to bulk $[H^+]$. This is consistent with the first order relation between bulk $[H^+]$ and the fluorescence transition observed between the second and the third flash in a series.

This model can be explained by several equilibria, following the basic principles outlined by McPherson et al. [42] and Maroti and Wraight [23]. The release of one principal proton at each flash can be described by the reaction:

$$\text{excitedcenter-H} + \text{DPC} \rightleftarrows \text{center-DPC}^+ + H^+.$$

Because of the large pK shift at each flash induced by the large energy involved in the oxidation of the electron donor, the equilibrium is strongly displaced. This proton release is better described as a complete irreversible release independent of pH. In contrast, the pH-dependent proton release/uptake induced by much smaller electrostatic effects can be described by pK shifts of the following equilibria for the bound H^+ and OH^- ions and water molecules at the bulk surface junction of the catalytic site:

$$H_2O_{bound} \rightleftarrows H_2O_{liquid} \rightleftarrows H^+_{liquid} + OH^-_{liquid}$$
$$\rightleftarrows H^+_{bound} + OH^-_{liquid} \rightleftarrows H^+_{liquid} + OH^-_{bound}$$

It results from these equilibria that there are 3 pK values for each of the 3 bound water molecules (the p$K = 7$ of liquid water is well known). As explained above, the release or uptake of the principal proton after each flash could alter the electrostatic interactions between the polarized catalytic center and the bound ions, thereby affecting the pK values. As indicated in Ref. [42], the shift in pK is also related to the change in position or value of the charges. The pK shift is approx. 2 for a distance of 5 Å between a proton and a negative charge and 1 for a distance of 7 Å [42]. These distances are sufficiently large to allow the presence of several water molecules in the place of the missing four manganeses. In O_2-evolving centers with the manganese cluster at the catalytic site, only one half water molecule is bound, as shown in the experiments of Haumann and Junge [39], instead of three water molecules in manganese-depleted centers. This difference could be due to the available space for water molecules around the reactive site in the pocket.

The electron donor is not shown in Scheme 1. In photoactivation, the first photooxidative event results in oxidation of one Mn^{2+} to Mn^{3+} and the second photochemical step is like the first and consists of photooxidation of a second Mn^{2+} [16]. By analogy with the electron donation reactions in Mn-depleted samples, it is inferred that two DPC molecules are photoooxidized before they are finally reduced for hydrogen peroxide formation.

There is some indication [43] that hydrogen peroxide is not formed in abundance in Mn-depleted PS II membranes in the presence of either Mn^{2+} or DPC as an electron donor. However, Fine and Frasch [44] found that the rate of hydrogen peroxide production generated upon illumination of O_2-evolving PS II core preparations increased with pH from pH 6.8 to 7.2, and is maximal at pH 7.2. The increased yield of hydrogen peroxide follows the pH dependence of centers competent in the two-photostep process, shown in Fig. 4 of this paper. This suggests that hydrogen peroxide could be effectively formed in Tris-treated PS II membranes in the presence of DPC, but not immediately released into the aqueous phase, as already proposed for lauroylcholine chloride-treated PS II membranes [45]. On the grounds that in the O_2-evolving Mn cluster di-μ-oxo bridging oxygen atoms are present [46], the storage of H_2O_2 is also expected to occur during photoactivation and to take part in assembly and binding the Mn cluster.

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