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Cofactor X of photosynthetic water oxidation: electron transfer, proton release, and electrogenic behaviour in chloride-depleted Photosystem II

Monika Hundelt, Michael Haumann, Wolfgang Junge *

Abteilung Biophysik, FB Biologie / Chemie, Universität Osnabrück, Barbarastr. 11, D-49076 Osnabrück, Germany

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Abstract

Four quanta of light, absorbed by Photosystem II (PS II), drive the catalytic center of oxygen evolution (OEC) through five transitions which are named $S_0 \Rightarrow S_1$ to $S_3 \Rightarrow S_4 \rightarrow S_0$ [1]. Manganese (Mn_4), tyrosine (Y_Z) and a chemically ill-defined compound, X, serve as redox cofactors. Transient optical absorption spectra of PS II core particles have led us to propose that the same cofactor X is oxidized on $S_2 \Rightarrow S_3$ in controls and on $S_1^* \Rightarrow S_2^*$ in Cl^--depleted centers [2]. In this work this particular transition was scrutinized by monitoring UV-transients, proton release and transmembrane electrochromism, both in Cl^--depleted and in control thylakoids. The oxidation of X by Y_Z^{ox} caused biphasic proton release: the fast component ($t_{1/2} \approx 35$ μs) was attributable to electrostatically induced pK-shifts of peripheral amino acid residues. It was transient and disappeared concomittantly with the rise of the slow component ($t_{1/2} \approx 220$ μs) that was attributed to proton liberation from X itself. The stoichiometric extent of 'chemical' proton release per $X^{\cdot}$ was 1:1. The transfer of a proton from X into the lumen of thylakoids was electrogenic with a relative extent of 10% of the one attributable to the formation of the charge pair Y_Z^{ox}/Q_A^-. The oxidation of X by Y_Z^{ox}, proton release and the 10% rise of the transmembrane voltage were all characterized by the same half-rise time of 220 μs. We propose that the membrane embedded X, after its oxidation and deprotonation during $S_2 \Rightarrow S_3$, serves as the postulated hydrogen acceptor during the final oxygen evolving step $S_3 \Rightarrow S_4 \rightarrow S_0$. © 1997 Elsevier Science B.V.

Keywords: Cl^--depletion; Photosystem II; Proton release; Electron transfer; Electrochromism; Water oxidation

Abbreviations: B^-, base form of amino acid; BBY, Photosystem-II enriched membrane fragments; BCP, bromocresol purple; BSA, bovine serum albumin; DCBQ, 2,5-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)1,1-dimethylurea; DNP-INT, dinitrophenol ether of iodonitrotoluol; EPR, electron paramagnetic resonance spectroscopy; Fwhm, full width at half maximum; Mn, manganese; OEC, oxygen-evolving complex = Mn_4X-entity; P_{680}, primary donor of PS II; PS II, Photosystem II; Q_A, primary quinone acceptor; Q_B, secondary quinone acceptor; S_i, *i*th oxidized state of the Mn_4XY_Z-entity; S_i^*, modified S-state; X, cofactor of the OEC; Y_Z, Y_D, redox active tyrosines 161 on subunits D1 and D2, $Y_z^{ox} = Y_Z^{\cdot} H^+ - B^-$, oxidized tyrosine

* Corresponding author. Fax: +49 541 9692870; E-mail: junge@uni-osnabrueck.de

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1. Introduction

Photosystem II (PS II) of cyanobacteria and green plants oxidizes water to dioxygen at the expense of four quanta of light. Electron transfer proceeds from the singlet excited primary electron donor (P_{680}^{*}) via pheophytin to the primary quinone acceptor Q_A and further on to Q_B. The secondary electron donor, tyrosine 161 on D1 (Y_Z), mediates the reduction of P_{680}^{+} by the oxygen-evolving complex (OEC) at the donor side of PS II. The Mn-containing catalytic center cycles through five oxidation states S_i (i: 0, 1,...,4), where i denotes the number of accumulated oxidizing equivalents in the Mn_4XY_Z-entity [1]. State S_1 is stable in the dark so that oxygen evolution occurs after the third saturating flash in a series, on $S_3 \Rightarrow S_4 \rightarrow S_0$. It is generally accepted that transitions $S_0 \Rightarrow S_1$ and $S_1 \Rightarrow S_2$ represent the oxidation of Mn [3,4]. The component that is oxidized on $S_2 \Rightarrow S_3$ is probably not Mn [3,4].

Chloride (Cl^-) is an essential cofactor of oxygen evolution. In its absence only two charges can be stably stored at the donor side, and from the third flash on the charge pair P_{680}^{+}/Q_A^{-} recombines in about 100 μs [5–7]. The nature of the components which are oxidized during the first two transitions in Cl^--depleted material is still under debate. Studies on Cl^--depleted materials in several laboratories and with various techniques have led the respective authors to conflicting concepts on the sequence of redox reactions [5,8–13]. The apparent discrepancies have sometimes been attributed to the use of different protocols for Cl^--depletion and to different starting materials, i.e. thylakoids, BBY-membranes and oxygen evolving core particles. In our previous work we studied the effects of Cl^--depletion mainly in core particles [2]. In the present work we collected all data from thylakoids. Our procedure for Cl^--depletion in thylakoids, the more native PS II material, was quite effective but rather gentle, as judged from a high rate of oxygen evolution after addition of Cl^-. With Cl^--depleted thylakoids we reproduced the result from our previous experiments with core particles, namely that the same component, X, was oxidized after the first flash in the absence of Cl^- ($S_1^{*} \Rightarrow S_2^{*}$) as after the second flash in controls ($S_2 \Rightarrow S_3$) [2]. In addition we characterized two properties of the oxidation of X, that were only accessible in thylakoids: (1) The extent and rate of proton release into the lumen of thylakoids was detected by the amphiphilic dye neutral red [14–16] and its origin from two sources, namely from the redox cofactor X itself ('chemical production') and from peripheral amino acids (electrostatic repulsion) was discriminated on kinetical grounds (for a review on this technique see [17]). (2) The transmembrane electrogenicity of electron and proton transfer was monitored by electrochromism [18,19].

Our data showed that the oxidation of X produced an electroneutral radical ($X^{\cdot}$) by virtue of the ejection of a proton from X into the thylakoid lumen. This result was in line with the particularly large kinetic H/D-isotope effect on the electron transfer from X to Y_Z^{ox} [20].

2. Materials and methods

Unstacked thylakoids were prepared from 12 days old pea seadlings (*Pisum sativum*) as described in [21] with Na_2SO_4 instead of NaCl in all media. They were frozen at −80°C in 1 mM Tricine/NaOH pH 7.8, 5 mM Na_2SO_4, and 0.4 M sucrose at ≤ 2 mM chlorophyll until use. *For Cl^--depletion* thylakoids were thawed and suspended at 80 μM chlorophyll in buffer A (5 mM Tricine/NaOH pH 7.8 and 5 mM Na_2SO_4) and gently stirred for 20 min on ice in the dark. They were collected by centrifugation (30 000 × g, 10 min, 4°C) and resuspended in buffer A to about 2.5 mM of chlorophyll. This method of Cl^--depletion was optimized (see also [2]) for obtaining high reversibility (near maximum oxygen rate in Cl^--repleted material, see below) and a large proportion of Cl^--free centers (≥ 60%). Cl^--depleted thylakoids, that were repleted with Cl^- before the measurements, served as controls. *Cl^--repletion* was achieved by incubating the Cl^--depleted thylakoids (30 μM) in the measuring buffer containing 20 mM NaCl for 15 min at room temperature. The whole procedure was carried out in complete darkness.

Oxygen evolution was measured under continuous white light illumination with a Clark-type electrode at 30 μM chlorophyll (21°C) in a medium which contained 10 mM Hepes/NaOH pH 7.2, 10 mM Na_2SO_4 (Cl^--depleted) or 20 mM NaCl (control), 2 mM ferricyanide, and 1 μM nigericin. Under continuous

light Cl^--depleted thylakoids evolved oxygen at a rate of about 20% of the control (= repleted material, about 130 μmol O_2/mg Chl per h). This rate was not limited by electron transfer between PS II and PS I because ferricyanide at the used high concentration (2 mM) acted as an effective electron acceptor already at PS II [22]. The turnover of PS I was $\leq 10\%$ of total (data not shown). Under single flashes (see Section 3), however, the percentage of active centers was larger, 40%. This result was understood if some centers which still contained Cl^- even after the procedure of Cl^--depletion lost Cl^- during continuous illumination in a Cl^--free medium. This may indicate that Cl^- is more weakly bound in S_0. The fraction of centers which was irreversibly inhibited by Cl^--depletion was about 10% as estimated from a comparison with untreated thylakoids (150 μmol O_2/mg Chl per h).

For flash-spectrophotometric measurements [23] thylakoids were suspended at 30 μM chlorophyll in 10 mM Na_2SO_4 or 20 mM NaCl, 2.6 g/l BSA, 15 μM DNP-INT, 2 mM ferricyanide, and 300 μM imidazole, and dark-adapted for 15 min at room temperature. Under these conditions PS I was oxidized to greater than 95% before the first flash of light [24]. For measurements with Cl^--depleted thylakoids BSA (20 g/l) was dialysed against 10 mM Na_2SO_4 for 8 h at room temperature to remove bound Cl^-. Further additions are indicated in the figure legends. The first exciting flash in a series was given by a Q-switched Ruby laser (694 nm, fwhm 50 ns), and the following flashes by a Xenon flashlamp (Schott RG610, fwhm 10 μs). With dark-adapted material every train of flashes was recorded with a fresh sample which was filled automatically into the cuvette from a light-shielded reservoir. Transients were digitized on a Nicolet Pro92 recorder. Up to 300 transients were averaged to improve the signal-to-noise ratio. The optical pathlength was 1 cm. *Electron transfer from the OEC to Y_Z^{ox}* was recorded at 360 nm as described [25] with 10 mM Tricine (pH 7.1) instead of BSA, 200 μM DCBQ, and 1 mM ferricyanide. The substitution of ^2H_2O for H_2O was performed before the measurements by suspending Cl^--depleted thylakoids in ^2H_2O at 30 μM chlorophyll followed by dark-adaptation for 15 min. The final concentration of ^2H_2O was 97%. *Proton release into the lumen* was detected at 548 nm with 30 μM of the amphiphilic pH-indicating dye neutral red. Background transients were recorded under the same conditions without the dye and subtracted ($\pm$dye, [26]). Proton release was time resolved with 20 μs/address at pH 7.2 and 6.1. *Proton uptake at the acceptor side* of PS II at pH 6.1 was measured by absorption changes of the hydrophilic dye bromocresol purple (BCP) at 575 nm ($\pm$dye) in the absence of BSA with a time resolution of 1 ms/address. *Electrochromic absorption transients of carotenoids at 522 nm* served to monitor the flash induced generation of transmembrane potential. Background transients at this wavelength were recorded in the presence of 500 nM gramicidin, which accelerated the decay of the transmembrane potential to a half-decay time of about 20 μs, and subtracted [18,19]. The resulting transients were solely due to transmembrane electrochromism.

3. Results

3.1. The rate of reduction of Y_Z^{ox} by X is similar on $S_1^* \Rightarrow S_2^*$ ($-Cl^-$) and on $S_2 \Rightarrow S_3$ ($+Cl^-$)

Fig. 1 shows absorption transients at 360 nm which reflect the electron transfer from the OEC to Y_Z^{ox} upon the first flash in dark-adapted Cl^--depleted thylakoids and controls in H_2O and ^2H_2O. The transients upon the second flash are not shown, but see [2,27]. The first flash which mainly induced transition $S_1 \Rightarrow S_2$ in controls ($+Cl^-$), caused an unresolved jump (indicated by the dashed line in Fig. 1, top). It was due to the formation of Y_Z^{ox}/Q_A^- in nanoseconds [28]. In the presence of Cl^- and in H_2O (Fig. 1, top, $+Cl^-$, solid circles) the jump was followed by a slower rise with a half-rise time of 55 μs (Table 1) which was attributable to the oxidation of Mn by Y_Z^{ox}. This half-rise time was similar to the one previously found in core particles on $S_1 \Rightarrow S_2$ [2,27]. In ^2H_2O (Fig. 1, $+Cl^-$, open circles) the half-rise time increased only slightly, to 75 μs ($k_H/k_D = 1.2$, Table 1), again in line with our previous results. In the absence of Cl^- (Fig. 1, bottom, $-Cl^-$) this picture was drastically changed: In H_2O (solid circles) the rapid jump on the first flash ($S_1^* \Rightarrow S_2^*$) was followed by a much slower rise than in the presence of Cl^- with a half-rise time of 220 μs (see legend of Fig. 1

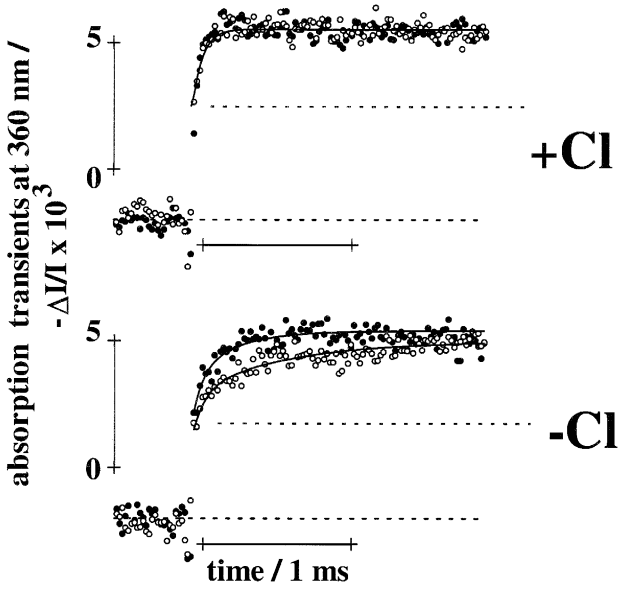


Fig. 1. UV-transients at 360 nm on the first flash in dark-adapted Cl^--depleted thylakoids and in controls. Upper traces: controls ($+Cl^-$): transients in H_2O (solid circles) and ^2H_2O (open circles). The line was calculated with $t_{1/2} = 55$ μs. Lower traces: Cl^--depleted material ($-Cl^-$): transients in H_2O (solid circles) and ^2H_2O (open circles). The lines were calculated with half-rise times of 220 μs (H_2O) and 480 μs (^2H_2O) and under the further assumption that 40% of centers, which still contained Cl^- (and therefore underwent transition $S_1 \Rightarrow S_2$), caused an admixture of components with $t_{1/2} = 55$ μs (H_2O) and 75 μs (^2H_2O, see Table 1) of control $S_1 \Rightarrow S_2$. Conditions: chlorophyll 30 μM, NaCl 20 mM (Cl^--depleted, Na_2SO_4 10 mM), Tricine 10 mM, pH 7.2, DCBQ 200 μM, ferricyanide 1 mM, 20 μs per address, Xe-flash, 220 transients were averaged.

and Table 1). We obtained a similar result in our previous work with Cl^--depleted core particles [2]. In ^2H_2O (Fig. 1, $-Cl^-$, open circles) the half-rise time greatly increased to 480 μs (Table 1). The kinetic H/D-isotope effect was $k_H/k_D = 2.2$. A similarly large H/D-isotope effect was obtained only on the second flash in the controls ($S_2 \Rightarrow S_3$) (here not documented, but see [20,27]). On the second flash in the absence of Cl^- (not shown) the jump was not followed by a rise, in line with the formation of metastable Y_Z^{ox} in nanoseconds [2]. We measured the rates of electron transfer on $S_1^* \Rightarrow S_2^*$ in Cl^--depleted material as function of the temperature between 4 and 30°C (data not shown). The activation energy as determined from an Arrhenius type plot of the rates was about 30 kJ/mol. It compares well with the activation energy of about 35 kJ/mol of transition $S_2 \Rightarrow S_3$ in active PS II [20,25].

We interpreted these results in line with our previous work on core particles: The rise on the first flash in the absence of Cl^- with the same half-rise time as on the second flash in the controls was attributed to the oxidation of one and the same species X by Y_Z^{ox} [2]. That the H/D-isotope effect and the activation energy of electron transfer on $S_1^* \Rightarrow S_2^*$ in the absence of Cl^- were about as large as on $S_2 \Rightarrow S_3$ in controls [20,25,27,29] corroborated this notion.

3.2. One proton per electron was released on $S_1^* \Rightarrow S_2^*$ and on $S_2 \Rightarrow S_3$ independent of the pH

Proton release in a train of flashes was recorded with the dye neutral red in dark-adapted Cl^--depleted and control thylakoids. The resulting pH-transients on flashes one to five are shown in Fig. 2 (left column) at pH 7.2 (A) and pH 6.1 (B). The numbers of protons released per flash and PS II reaction center (right column of Fig. 2) were determined from the controls by setting the average extent of transients from flashes one to four to one proton per electron. In control samples (Fig. 2A,B, upper traces, left column) the raw patterns of proton release on the first four flashes were 0.75, 1.0, 1.25, 1.0 at pH 7.2 (Fig. 2A, right column, squares) and 2.1, 0.8, 0.45, 0.65 at pH 6.1 (Fig. 2B, right column, squares). These patterns were similar to previous ones in the same type of material [16]. The extent after the first flash was here probably slightly overestimated because of the contribution of centers that were irreversibly inhibited by Cl^--depletion and -repletion (about 10%, see Section 2). In contrast to the pH-dependent extent on the first flash ($S_1 \Rightarrow S_2$) in controls, proton release on $S_1^* \Rightarrow S_2^*$ in Cl^--depleted thylakoids was pH-independent (Fig. 2A,B, left column, lower traces). The release of about one proton was observed after the first flash ($S_1^* \Rightarrow S_2^*$) both at pH 7.2 and 6.1 (Fig. 2A,B, right column, circles). A pH-independent release of about one proton was also observed after the second flash ($S_2 \Rightarrow S_3$) in control thylakoids [16,30]. In Cl^--depleted samples about 0.4 protons were still released from the third flash on. They were attributed to 40% of PS II centers which still contained Cl^- and were active in oxygen evolution.

Table 1
Comparison of the half-rise times of the reduction of Y_Z^{ox}, of proton release, and of the electrogenic components from dark-adapted Cl^--depleted thylakoids ($-Cl^-$) and controls ($+Cl^-$)

			Thylakoid material:		
			controls		Cl^--depleted
			transition:		
			$S_1 \Rightarrow S_2$	$S_2 \Rightarrow S_3$	$S_1^* \Rightarrow S_2^*$
Observable:	pH	medium	half − rise time (μs)		
Electron transfer (360 nm)	7.2	^2H_2O	75	450 [a]	480
		H_2O	55	220 [a]	220
Electrochromism (522 nm)			/ [c]	200 (13%) [d]	220 (10%) [d]
Proton release (548 nm)			35 (0.5) [b]	220 (1) [f] 35 (1) [e]	220 (1) 35 (0.5) [e]
	6.1		35 (2.0)	220 (1) [f] 35 (1) [e]	220 (1) 35 (2.0) [e]

[a] These half-rise times were taken from [27]; [b] figures in parentheses give the extents of proton release; [c] an electrogenic component on this transition was here not resolved; [d] the raw electrogenic amplitudes in % of Y_Z^{ox}/Q_A^--formation; [e] these protons were rebound upon reduction of Y_Z^{ox} by X; [f] these protons are masked by the periphery and not directly observable.

Under the low salt conditions (only 20 mM NaCl or 10 mM Na_2SO_4) in the above experiments, the negative surface charge at the lumenal side of the thylakoid membrane may vary in the presence and absence of Cl^- thereby causing a redistribution of neutral red at the membrane [31]. As a control, proton release was recorded again under high salt concentrations (plus 20 mM $MgCl_2$ or $MgSO_4$) at pH 6.7 (Fig. 2C). The high concentration of cations screens the surface potential and eliminates any salt induced redistribution of neutral red [31]. The observed proton pattern of 0.55, 1.2, 1.45, 0.8 in control thylakoids and the release of one proton after the first flash in Cl^--depleted centers (Fig. 2C) resembled the features that were observed under low salt conditions (Fig. 2A). Accordingly, these features were not affected by the detection method.

The *pH-independent* release of one proton per electron on $S_1^* \Rightarrow S_2^*$ (first flash, $-Cl^-$) and $S_2 \Rightarrow S_3$ (second flash, $+Cl^-$) was interpreted as showing the 'chemically' produced proton during the oxidation of X. Accordingly, it was expected that the electrostatical situation in the OEC remained unchanged during $S_2 \Rightarrow S_3$ (and $S_1^* \Rightarrow S_2^*$). The variable and *pH-dependent* release of 2.1 protons (pH 6.1) and 0.75 protons (pH 7.2) on $S_1 \Rightarrow S_2$ (first flash, $+Cl^-$), on the other hand, was caused by the electrostatically induced deprotonation of *peripheral groups*. Upon the metastable formation of Y_Z^{ox} on the second flash in Cl^--depleted centers we expected the same extent of proton release from peripheral groups as observed on $S_1 \Rightarrow S_2$ in the controls ($+Cl^-$). The patterns of proton release in Cl^--depleted centers that were calculated under the assumption of a 40%-contribution by active centers (see above) are shown in Fig. 2 as asterisks. They approximately reproduced the observed experimental patterns in Cl^--depleted centers (open circles). This result corroborated that the pH-dependences of the extents of proton release from peripheral groups were the same in Cl^--depleted centers and in controls.

3.3. The rates of proton release in Cl^--depleted centers and in controls

Proton release was time resolved after the first flash in dark-adapted thylakoids at pH 7.2 and pH 6.1 (Fig. 3). We aimed at a discrimination between proton release from peripheral amino acids and chemical production of protons from the catalytic center. Whereas the former can be faster than Y_Z^{ox}-reduction, the rate of the latter coincides with the one of the electron transfer to Y_Z^{ox} (for reviews see [17,30]).

The upper traces in Fig. 3 show absorption transients of neutral red at pH 7.2. In the control (left) 0.75 protons were released with a half-rise time of 35 μs (solid line). In Cl^--depleted material (right) the pH-transient was described by two exponentials (solid line) with half-rise times of 35 μs (0.75 protons, indicated by the dashed line) and 220 μs (0.35 protons). Taking into account that 40% of centers were still active (see above) the extent of the 220

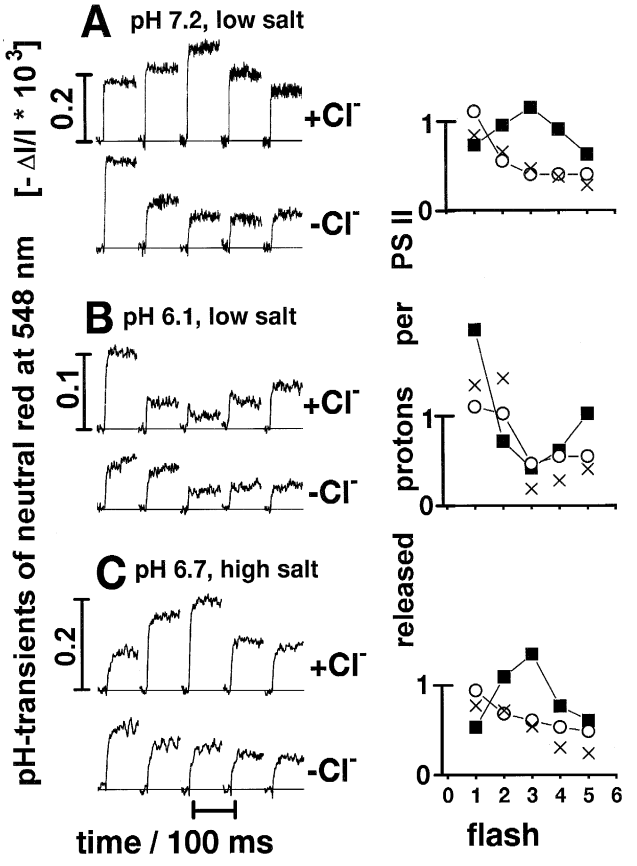


Fig. 2. Flash-induced absorption transients (left column) of the pH-indicator neutral red (±dye 20 μM) at 548 nm from dark-adapted, Cl^--depleted thylakoids (lower traces) and controls (upper traces). The patterns of proton release as function of the flash number (right column) in Cl^--depleted thylakoids (open circles) and in controls (squares) were calculated from the absorption transients in the left column. The time resolution was 1 ms per address, 30 transients were averaged. The asterisks in A–C (right column) denote the theoretical patterns of proton release that were calculated under the assumptions outlined in the text and with a contribution of 40% active centers. A: pH 7.2, low salt conditions: 10 mM Na_2SO_4 or 20 mM NaCl, flash spacing 100 ms. B: pH 6.1, low salt conditions as in A, flash spacing 100 ms. C: pH 6.7, high salt conditions: 10 mM Na_2SO_4 plus 20 mM $MgSO_4$ or 20 mM NaCl plus 20 mM $MgCl_2$, the spacing between flashes was 200 ms.

μs-rise recalculated to 0.5 protons. In the bottom row of Fig. 3A the observed kinetics were subdivided into proton release from peripheral amino acids and from the catalytic center ('chemically' produced protons). In the control (left) proton release occured only from peripheral groups (dashed-dotted line). In Cl^--depleted material the same amount of protons was released by peripheral groups in the presence of Y_Z^{ox} and these protons were rebound when the electron hole was transferred further on to X (dashed-dotted line). The oxidation of X caused the release of one chemically produced proton (dotted line). The sum of these components is shown as a solid line in the top trace of Fig. 3A.

Fig. 3B shows pH-transients of neutral red at pH 6.1. At this pH it was not possible to use the same combination of 100 μM ferricyanide plus DCMU as at pH 7.2. Because the pH-transients at pH 6.1 were much smaller than at pH 7.2, due to the pH-dependent sensitivity of the dye [16], it was necessary to fully oxidize P_{700} by a higher concentration of ferricyanide, 2 mM, in order to avoid artifacts in the procedure of taking differences of small transients (±dye). 2 mM ferricyanide oxidized the non-heme iron in the dark and led to fast proton uptake from the suspending medium upon the reduction of $Fe^{(III)}$ on the first flash [32] which was more slowly buffered by BSA. The pH-transients of neutral red at pH 6.1 from the donor side were thus superimposed by transients from the acceptor side [15]. In control material (Fig. 3B, upper trace, left) this superimposition caused the slow rising component ($t_{1/2} = 3$ ms, 65%) on the pH-transient after the first flash. A minor component (35%) rose with $t_{1/2} \approx 35$ μs. The lower row of Fig. 3B (left) shows the extents of the individual protolytic components: (1) the fast release of two protons from peripheral amino acids with $t_{1/2} \approx 35$ μs (dashed-dotted line) was superimposed by (2) the uptake of protons at the acceptor side upon the reduction of the non-heme iron followed by the buffering of this alkalization by BSA in 3 ms (dashed line). The sum of these components resulted in the solid line in the upper row (left) of Fig. 3B.

In Cl^--depleted centers the slowly rising component due to protolytic events at the non-heme iron was apparently absent (Fig. 3B, top, right). We checked whether proton uptake at the PS II acceptor side was similar in Cl^--depleted centers and controls: Fig. 4 shows transients of the hydrophilic dye bromocresol purple, indicating proton uptake at the acceptor side of PS II, in dark-adapted thylakoids on the first five flashes. The extent of proton uptake on the first flash was similar in Cl^--depleted thylakoids and in controls. From the third flash on the extent of proton uptake in the absence of Cl^- was only about 40% of the controls. The latter result corroborated the

notion that 40% of PS II in Cl^--depleted samples were still active. On the basis of these results the most likely interpretation of the lack of the slowly rising component on the first flash in Cl^--depleted material (Fig. 3B, top, right) was that it was compensated by *proton uptake at the donor side*. The se-

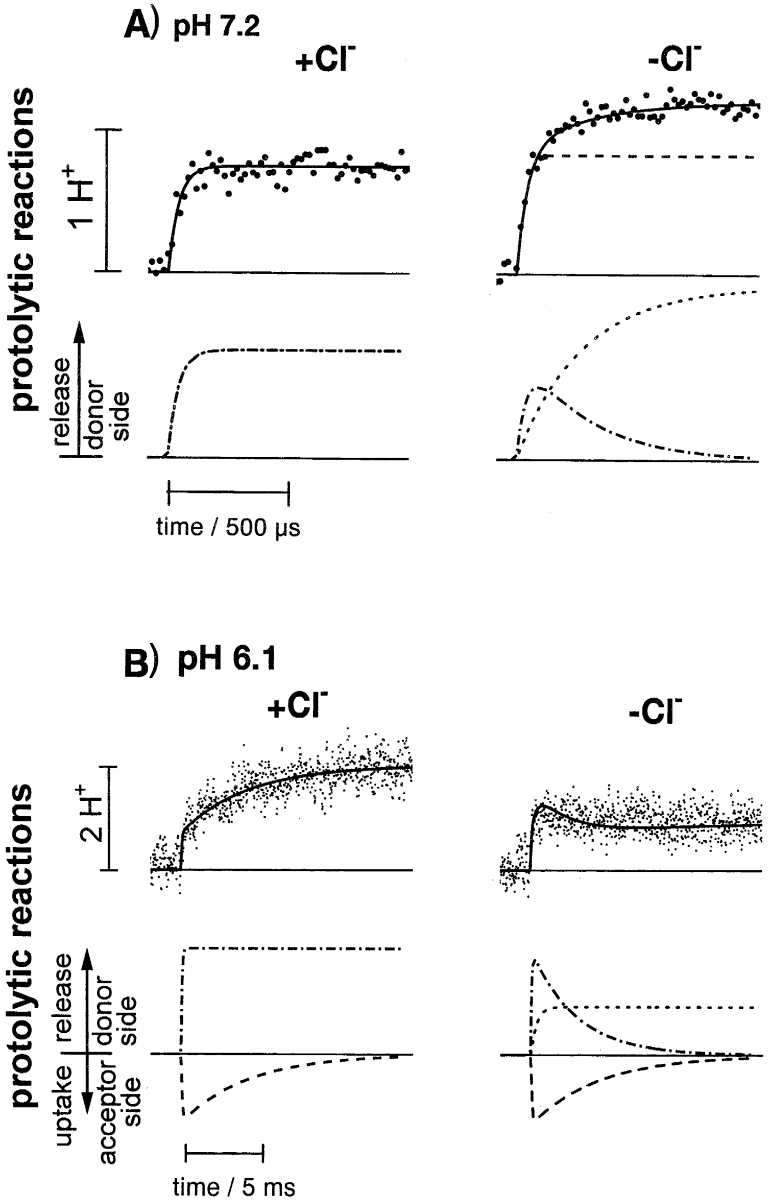


Fig. 3. Time-resolved pH-indicating absorption transients of neutral red (±dye 20 μM) at 548 nm after the first flash in dark-adapted, Cl^--depleted thylakoids (right column) and controls (left column). The time resolution was 20 μs per address, 100–300 transients were averaged. The amplitudes that corresponded to one proton were calculated from Fig. 2. A: upper row: raw data at pH 7.2, 20 μM DCMU and 100 μM ferricyanide were added. B: upper row: raw data at pH 6.1, 2 mM ferricyanide and no DCMU were added. Note the different time scale as in A. A,B, lower rows: calculated time courses of the protolytic components that contributed to the observed pH-transients. Dashed-dotted lines: proton release from peripheral amino acids at the PS II donor side with $t_{1/2} \approx 35$ μs, at pH 6.1. B: these protons are rebound with $t_{1/2} = 2$ ms; dotted lines: 'chemical' proton production from $X^{\cdot}$ with $t_{1/2} = 220$ μs; dashed line: transient superimposition of rapid proton uptake at the PS II acceptor side upon the reduction of the non-heme iron, which is subsequently buffered by BSA in the medium with $t_{1/2} = 3$ ms. The respective sum of the calculated components is shown as a solid line in the upper rows of A and B. For further details, see text and Scheme 2.

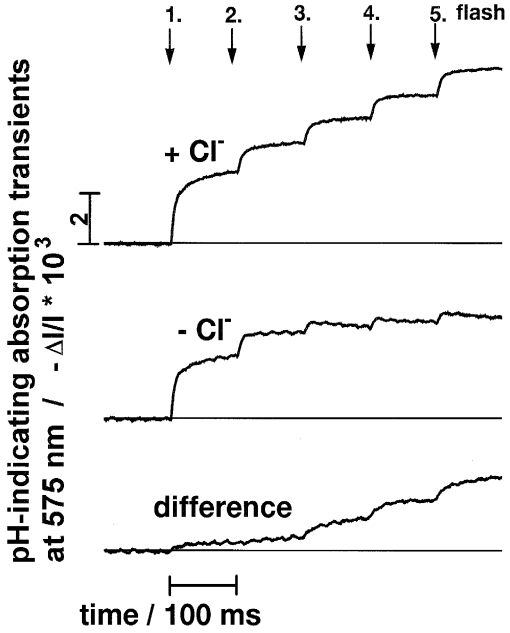


Fig. 4. Proton uptake from the medium at the PS II acceptor side as indicated by the pH-transients of bromocresol purple ($\pm$dye) on the first five flashes in dark-adapted, Cl^--depleted thylakoids (middle) and in controls (top) at pH 6.1. The difference ($\pm Cl^-$) is shown in the bottom trace. Conditions: 2 mM ferricyanide, time resolution 1 ms per address, 100 transients were averaged.

quence of protolytic events at pH 6.1 ($-Cl^-$) is shown in the bottom row (right) of Fig. 3B: (1) the fast release of two protons at the donor side from peripheral groups in 35 μs upon Y_Z^{ox}-formation was followed by their re-uptake in 2 ms when Y_Z^{ox} was reduced by X (dashed-dotted line). The slow re-uptake of protons from peripheral groups in milliseconds has been previously observed on $S_4 \rightarrow S_0$ in thylakoids at pH 6.3 [16]. (2) These events were superimposed by the fast uptake of protons at the acceptor side and the buffering of this alkalization in 3 ms (dashed line). (3) The release of one chemically produced proton from the catalytic center occured with $t_{1/2} \approx 220$ μs (dotted line). The solid line in the upper row of Fig. 3B (right) represents the sum of these components. It was in line with the experimental data. We concluded that both at pH 7.2 and pH 6.1 one proton was released on $S_1^* \Rightarrow S_2^*$ (first flash, $-Cl^-$) with the same half-rise time (about 220 μs) as the electron transfer from X to Y_Z^{ox}.

3.4. The electrogenicities of proton release on transitions $S_1^* \Rightarrow S_2^*$ and $S_2 \Rightarrow S_3$

Fig. 5 shows transients of transmembrane electrochromism at 522 nm in dark-adapted, Cl^--depleted thylakoids and controls on the first (left) and the second flash (right). These transients are indicative of charge transfer in the direction of the membrane normal [18]. The formation of Y_Z^{ox}/Q_A^- in ≤ 1 μs [33] appeared as an unresolved stepped rise (Fig. 5). In the presence of gramicidin the decay of the electrochromic response to the flash-induced transmembrane potential was accelerated to a half-rise time of about 20 μs (data not shown), whereas the electrochromic response to local charges in PS II remained the same as in the absence of gramicidin.

On the first flash (Fig. 5, left, top) the extent of the electrochromic transient after 1 ms in the presence of Cl^- was smaller than in its absence. On the second flash its sign was reversed. We took the differences $\pm Cl^-$ (Fig. 5, bottom traces). They were described by single exponentials (solid lines) with respective half times of 220 μs (first flash) and 200 μs (second flash) (Table 1). It was noteworthy that the traces in the presence of Cl^- were obtained after adding Cl^- back to depleted samples. The above results were interpreted as follows: On the first flash in Cl^--depleted thylakoids the reduction of Y_Z^{ox} by X ($S_1^* \Rightarrow S_2^*$) contributed an electrogenic rise with $t_{1/2} \approx 220$ μs (Fig. 5, left, bottom). On the second flash in Cl^--depleted samples Y_Z^{ox}/Q_A^- was formed and Y_Z^{ox} remained stable [2,10]. In the control Y_Z^{ox} was reduced by X in about 200 μs in an electrogenic reaction (Fig. 5, right, bottom). The half-rise time of the additional electrogenic components was about 220 μs both on $S_1^* \Rightarrow S_2^*$ (first flash, $-Cl^-$) and on $S_2 \Rightarrow S_3$ (second flash, $+Cl^-$). It coincided with the one of Y_Z^{ox}-reduction as measured in the UV (see Fig. 1). The same half-rise time was observed for proton release on $S_1^* \Rightarrow S_2^*$ (see Fig. 3). The amplitude of the electrogenic rise on $S_1^* \Rightarrow S_2^*$ was 6% of the jump after the second flash in the absence of Cl^-, which was due to formation of Y_Z^{ox}/Q_A^- [2]. Taking into account that 40% of PS II-centers were still fully active in the Cl^--depleted material the observed extent of 6% was recalculated to yield 10% (Table 1). After the second flash the extent of the difference of transients $\pm Cl^-$ was 13% of Y_Z^{ox}/Q_A^--formation.

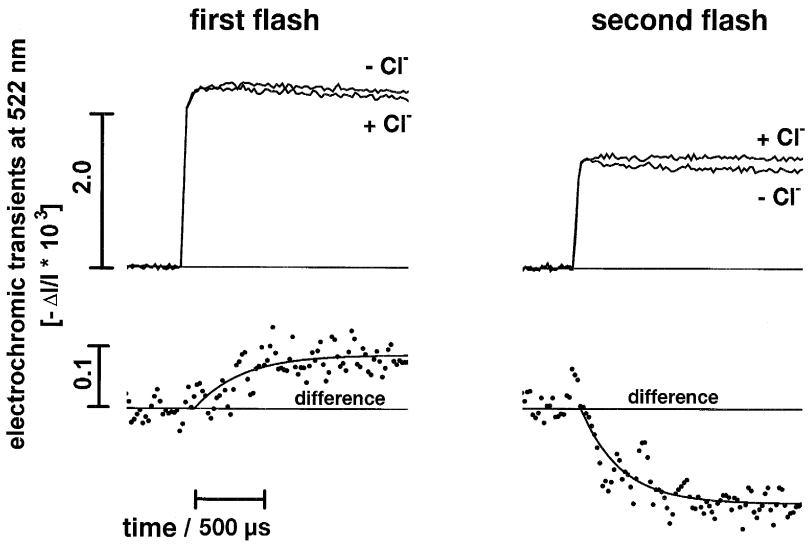


Fig. 5. Transmembrane electrochromism of carotenoids monitored at 522 nm after the first (left column) and second flash (right column) in dark-adapted, Cl^--depleted thylakoids ($-Cl^-$) and in controls ($+Cl^-$) at pH 7.2. The traces represent the differences of transients $\pm$500 nM gramicidin. The differences ($-Cl^-$) minus ($+Cl^-$) are shown in the bottom row. The solid lines correspond to single exponentials with half-rise times of 220 µs (first flash) and 200 µs (second flash).

The amplitude that exceeded 10% was caused by a small kinetic phase from centers that underwent charge recombination (in about 100 µs) between P_{680}^+/Q_A^- already on the second flash due to the double hit factor. The latter event also seemingly decreased the half-rise time to 200 µs instead of 220 µs.

4. Discussion

4.1. The first two flashes given to Cl^--depleted PS II cause the sequential formation of $X^\wedge$, Y_Z^{ox} both in thylakoids and PS II core particles

In the absence of Cl^- only two charges can be stably stored at the PS II donor side [5,6]. The sequence of oxidation steps has been debated among various authors [5,9–13]. Some discrepancies have been attributed to the use of different protocols of Cl^--depletion and of different PS II preparations as starting material. In this work we demonstrated that Cl^--depletion caused the same effects in thylakoids as previously found in oxygen evolving PS II core particles [2]: Electron transfer to Y_Z^{ox} on the first transition ($S_1^* \Rightarrow S_2^*$) in Cl^--depleted centers was much slower than on $S_1 \Rightarrow S_2$ in the control. The former occured with the same half-rise time as $S_2 \Rightarrow S_3$ of about 220 µs. Furthermore, the H/D-isotope effects and the activation energies of electron transfer were similar on $S_1^* \Rightarrow S_2^*$ and $S_2 \Rightarrow S_3$ ([20,29], and this work). These results strengthened our previous notion [2] that the same component, X, is oxidized on these two transitions. Cl^--depletion can thus be used as a tool to study the equivalent of transition $S_2 \Rightarrow S_3$ on the first flash after dark adaptation.

That the shift of the Mn K-edge of X-ray absorption was smaller on transition $S_2 \Rightarrow S_3$ than on $S_1 \Rightarrow S_2$ control material has been interpreted as indicating the absence of a Mn-oxidation on $S_2 \Rightarrow S_3$ in oxygen evolving PS II (for a review, see [34]). That Mn was not oxidized on the first transition in Cl^--depleted centers ($S_1^* \Rightarrow S_2^*$) has been infered from EPR work [9]. Contrastingly, a shift of the Mn K-edge of about 0.7 eV in Cl^--depleted centers on the first transition was interpreted as a Mn-oxidation [11]. The extents of both shifts as observed on $S_2 \Rightarrow S_3$ and on $S_1^* \Rightarrow S_2^*$ are, however, also compatible with structural changes [34] or the oxidation of a ligand to Mn.

We interprete our results as follows:

It is conceivable that the elimination of Cl^- increases the midpoint potential of manganese, by say, 300 mV [2]. As a result transition $S_0 \Rightarrow S_1$ still functions in Cl^--depleted material [35] whereas $S_1 \Rightarrow S_2$ is blocked. It is our view that Cl^--depletion raises the midpoint potential of Mn to such an extent that it cannot be anymore oxidized by Y_Z^{ox}. Accordingly, the terminal electron donor to Y_Z^{ox}, namely X, in Cl^--depleted centers has a higher midpoint potential than the one in controls (namely undisturbed Mn). This view is in seeming contrast with conclusions to the opposite which are based on thermoluminescence experiments. Vass et al. [36] have observed a lower peak temperature in Cl^--depleted centers as in controls (first flash) and have concluded that the midpoint potential of the electron donor in Cl^--depleted centers is more negative than in controls. However, this view is not unequivocal because peak temperatures are attributable to the charge-*pair* recombination. In the same work [36] the authors have presented data which clearly show that Cl^--depletion has a side effect on the acceptor side of PS II. The extents to which the peak temperatures changed upon Cl^--depletion are different for $S_2Q_A^-$ (10°C), $S_2^*Q_A^-$ (40°C) and $S_2Q_B^-$ (30°C), $S_2^*Q_B^-$ (45°C) [36]. It implies that the potential of Q_A is increased relative to the one of Q_B in Cl^--depleted centers.

On the second flash in Cl^--depleted material Y_Z is metastably oxidized [2,37]. Thus, on the first two transitions the two oxidized species that are formed are $X^{\cdot}$ and Y_Z^{ox}. This sequence of events is independent of the use of thylakoids or core particles and of the specific Cl^--depletion procedures that we applied to these materials.

4.2. The correlation between deprotonation and electron transfer

The reduction of Y_Z^{ox} by X was accompanied by the release of one proton per electron into the lumen. The stoichiometry was independent of the pH and the half-rise time was the same as the one of electron transfer. The proton is probably 'chemically produced' by X itself. Contrastingly, proton release due to the electrostatic interaction of peripheral groups with the charge on Y_Z^{ox} is pH-dependent. Y_Z^{ox} should perhaps be written as $Y_Z^{\cdot} \cdots H^+$-B with B standing for a nearby base (see [27] for details): It ranged from 0.75 protons at pH 7.2 to about two protons at pH 6.1 in Cl^--depleted centers and controls. This pH-dependence was similar to our previous results from control thylakoids [16]. We concluded that the electrostatic interactions between the peripheral groups and Y_Z were independent of the presence or absence of Cl^-.

The decrease of the rates of electron transfer to Y_Z^{ox} in higher state-transitions has been attributed to the increasing net-charge of the OEC [38,39]. On the other hand, the oxidation of X on transition $S_2 \Rightarrow S_3$ is electroneutral: the electron abstraction is likely steered by the proton transfer ([27] and this work). We propose that the different rates of electron transfer in the Kok-cycle rather indicate different *chemical* reactions: The oxidation of Mn [40] on $S_0 \Rightarrow S_1$ and $S_1 \Rightarrow S_2$ (in about 50 μs), the oxidation/deprotonation of X on $S_2 \Rightarrow S_3$ (in about 220 μs, this work), and the reduction of the OEC by electrons from water on $S_3 \Rightarrow S_4 \rightarrow S_0$ (in about 1 ms, for the respective half times see [25,27]). We propose that the electron transfer between X and Y_Z^{ox} is rate-limited by 'chemical' proton release from X because of its larger kinetic H/D-isotope effect ([20] and this work). The deprotonation of X may decrease the redox-potential of the OEC according to experiments with model compounds [41]. The ability of X to eject one proton is likely pivotal for its oxidation by Y_Z^{ox}.

It has been proposed that bases that are formed in the lower S-transitions may accept a proton during $S_4 \rightarrow S_0$, thereby facilitating electron transfer upon the final oxidation of bound water [42]. According to our view, $X^{\cdot}$ (rather than Y_Z^{ox} [43,44]) is the prime candidate to accept a proton and an electron in concerted action.

Proton transfer from $X^{\cdot}$ into the lumen is more electrogenic than the electron transfer from Mn_4 to Y_Z^{ox} on transition $S_1 \Rightarrow S_2$. The electrogenicity of the former reaction is about 10% of the one caused by the formation of Y_Z^{ox}/Q_A^-. With a separation of the latter cofactors by about 31 Å [45] and under the assumption of a homogeneous dielectric an unrealistically small distance between X and Y_Z and the lumen is calculated. Under the more likely assumption of a higher dielectric permittivity between Y_Z and the lumen of, say, 40 (a similar value has been estimated for the Q_B binding site [46]) the latter distance yields

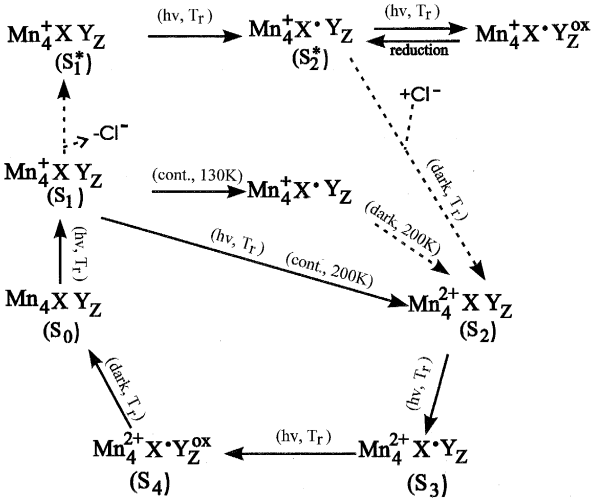
an upper limit of about 12 Å. This figure is compatible with the distance of 11 Å between Arg135, the analogue of Y_Z in the reaction center of purple bacteria, and the protein boundary [47]. In PS II the distance from Y_D to the boundary has been estimated as about 20 Å [48] and Y_D and Y_Z are supposedly symmetrically placed with respect to P_{680} [49]. Thus, about half of the distance between Y_Z and the lumen is probably covered by the extrinxic proteins. These proteins do probably not contribute to the electrogenicity of proton transfer in PS II in line with our recent results from electrometric measurements with PS II core particles [50].

4.3. On the nature of the cofactor X: our results compared to the literature

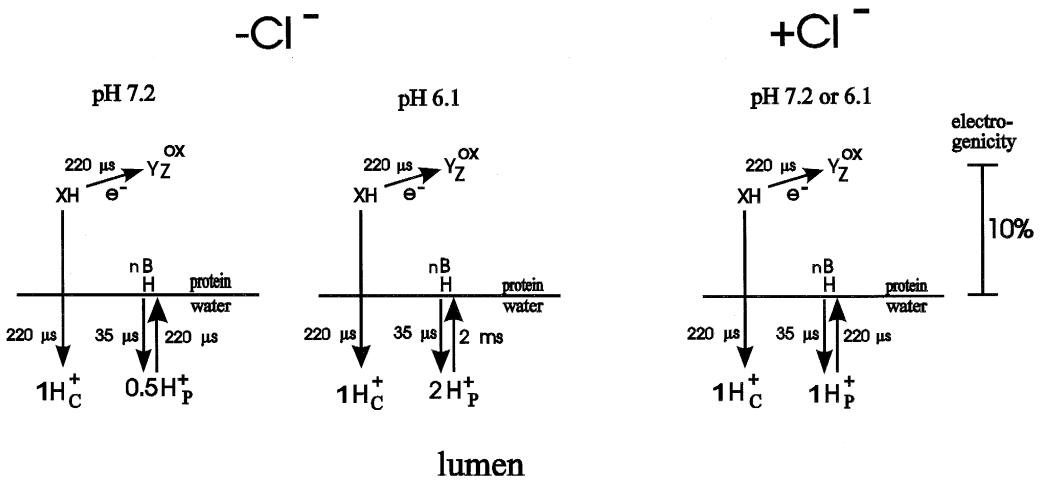
On the second transition in Cl^--depleted centers a split radical signal can be observed by EPR [9,13]. A similar signal has first been observed in calcium-depleted material [51]. The radical has been recently identified with Y_Z^{ox} [37,52]. This interpretation is in line with our previous results [2]. The splitting of the aforementioned radical signal (which differs from the EPR signal of Y_Z^{ox} in Tris-treated, i.e. Mn-depleted material [53]) has been attributed to the magnetic interaction between Y_Z^{ox} and a second paramagnetic entity at a distance of about 5 Å [54]. The latter component has been proposed to be the Mn-cluster from ENDOR data [55] in the S_2 state [12,56]. The estimation of a distance between Y_Z and Mn of about 15 Å by pulsed EPR [57] was apparently inconsistent with this view. Our experiments with Cl^--depleted material ([2] and this work) gave no evidence for the formation of the normal S_2-state of manganese on the first transition, again in line with the absence of the multiline EPR-signal [8]. According to our data the species that interacts with Y_Z^{ox} to yield the split radical signal may therefore be the cofactor X˙.

The nature of cofactor X is under debate. An EPR signal at $g = 4$, that appears upon the formation of X˙ on the first transition in Cl^--depleted centers [8,9], has been attributed to a spin 3/2 state [58]. The latter may be comprised of the Mn-cluster in S_1, supposedly of spin 1 [59], plus the spin 1/2 of a free radical species [60]. The $g = 4$ signal may thus be due to the state $Mn_4^+X˙Y_Z$.

The following properties of the $g = 4$ signal have been reported: (1) In acetate-treated centers the $g = 4$ signal rises in the dark with the same rate as the split radical signal (that is formed after two turnovers) decays [61]. (2) In Cl^--depleted samples that were preilluminated by one flash the $g = 4$ signal converts into the multiline signal in the dark after readdition of Cl^- [8]. (3) In control samples a $g = 4$ signal appears after one turnover at 130 K and converts into the multiline in the dark upon warming to 200 K [60,62]. These results are compatible with the following notions: (a) The $g = 4$ signal arises from the interaction between Mn in S_1 and X˙. (b) The split radical signal arises from the interaction between X˙ and Y_Z^{ox}. Thereby it is assumed that the state $Mn_4^+X˙Y_Z^{ox}$ is (1) reduced to $Mn_4^+X˙Y_Z$ and the latter decays into $Mn_4^{2+}XY_Z$ upon (2) Cl^--repletion or (3) warming. In O_2-evolving centers X is oxidized on $S_2 \Rightarrow S_3$ thereby yielding the EPR-silent state $Mn_4^{2+}X˙Y_Z$. On this transition the S_2-state multiline EPR signal, attributed to a spin 1/2 state [63] may disappear due to its coupling to X˙. We summarized these transitions in inhibited and control materials in Scheme 1. The



Scheme 1. Hypothetic sequences of oxidation/reduction in the oxygen-evolving and inhibited water oxidizing complex of PS II. $-Cl^-$: Cl^--depletion, $+Cl^-$: readdition of Cl^-, hν: one saturating flash of light, cont.: continuous illumination, T_r: room temperature, S_i: ith oxidation state of the Mn_4XY_Z-entity in control PS II, S_i^*: ith oxidation state of the Mn_4XY_Z-entity in Cl^--depleted PS II, solid arrows: oxidation/reduction of the Mn_4XY_Z-entity.



Scheme 2. The reactions of electron and proton transfer on transition $S_1^* \Rightarrow S_2^*$ in Cl^--depleted PS II and on $S_2 \Rightarrow S_3$ in controls (at pH 6.1 and 7.2). Electron transfer is denoted by open arrowheads, proton transfer by solid ones. Peripheral amino acids (nAH) released between 0.5 and 2 protons (H_P^+) upon formation of Y_Z^{ox}. The rebinding of these protons was steered by the reduction of Y_Z^{ox} by the cofactor X with a half-rise time of 220 µs at pH 7.2. At pH 6.1 proton rebinding was more slowly, $t_{1/2} = 2$ ms, as previously observed [16]. The oxidation of X was accompanied by the transfer of one 'chemically' produced proton (H_C^+) into the lumen. The electrogenicity of proton transfer from $X^{\cdot}$ to the lumen on $S_1^* \Rightarrow S_2^*$ was 10% of the one of Y_Z^{ox}/Q_A^--formation.

attribution of the $g = 4$ EPR-signal, however, is under contention (see [62]).

5. Conclusions

The reactions of electron- and proton-transfer on transition $S_1^* \Rightarrow S_2^*$ in Cl^--depleted centers and on $S_2 \Rightarrow S_3$ in controls are shown in Scheme 2: The same component X, is oxidized by Y_Z^{ox} on both transitions. The oxidation of X produces a neutral radical due to the pH-independent liberation of one proton per electron from $X^{\cdot}$. It is extruded into the aqueous phase at the lumen side. In thylakoids the proton transfer is electrogenic (distance < 12 Å). A likely function of $X^{\cdot}$ may be to accept a hydrogen-atom on the final oxygen-evolving transition $S_4 \rightarrow S_0$, which is expected to kinetically and thermodynamically facilitate electron transfer from bound water to the S_4-state ($Mn_4^{2+} X^{\cdot} Y_Z^{ox}$) of the OEC.

6. Note added in proof

After the acceptance of this article it came to our knowledge that the midpoint potential of Q_A was about equal (± 50 mV) in Cl^--depleted and control BBY-membranes as shown by redox titrations [64]. Furthermore, the thermoluminescence bands as obtained in Cl^--depleted and control materials appear at about the same temperature when samples are illuminated at room temperature before the measurements (A. Krieger, C. Jegerschöld, personal communications, manuscript in perparation). These results are well compatible with the same or a slightly higher midpoint potential of the couple S_2^*/S_1^* in Cl^--depleted centers as compared to S_2/S_1 in controls [2].

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