Photosynthetic oxygen evolution:
net charge transients as inferred from electrochromic bandshifts are independent of proton release into the medium

Michael Haumann, Oliver Bögershausen, Wolfgang Junge*

Abt. Biophysik, FB Biologie/Chemie, Universität Osnabrück, D-49069 Osnabrück, Germany

Abstract The manganese containing center of the oxygen evolving complex accumulates four oxidizing equivalents in the four stepped water oxidation cycle. Based on experiments on electrochromic absorption transients and the reduction rate of the primary electron donor, P_{680}, it has been speculated that the oscillations of these variables reflect the net charge of the center as calculated from the difference between electron abstraction and proton release into the medium. We compared proton release with electrochromism in thylakoids and core particles, and under variation of the rate of proton release. We found no equivalent of the variations of the extents and the rates of proton release in electrochromism. The oscillatory pattern of the latter reflects the topological properties of the stepped charge storage relative to the position and orientation of electrochromically responsive pigments rather than responding to proton release from the periphery.

Key words: Photosynthesis; Photosystem II; Proton release; Electron transfer; Electrochromism; Electrostatics; Net charge

1. Introduction

Four turnovers of photosystem II (PSII) produce dioxygen from two water molecules. Each of four absorbed quanta of light generates P_{680}^+ which oxidizes the intermediate carrier Y_Z (Tyr-161 on the D1 protein) [1]. This steps the manganese cluster from state S_0 (the most reduced state) to S_4, which spontaneously decays into S_0 under release of dioxygen. The distribution of the four protons over the redox transitions varies strongly depending on the pH and on the type of PSII preparation (see [2] for a review) whereas the pattern of oxygen evolution is invariant [3]. Under certain conditions the major portion of proton release occurs in some 10 μs, before the electron hole is deposited on the manganese cluster, i.e. at the level of Y_Z^+ [4]. Both the variability of proton release and its origin at the level of Y_Z^+ are indicative of an electrostatic origin, owed to pK shifts of peripheral amino acid residues in response to the positive charge deposited in the center. Chemically produced protons are only detected during the oxygen evolving transition $S_3 \Rightarrow S_4 \to S_0$, and only in thylakoids as the fractional slow proton release (alkaline pH) or uptake (acid pH) which complements the total number of preformed bases [4].

We asked whether the mainly peripheral nature of the detectable proton release during all four oxidation steps, $S_0 \Rightarrow S_1 \Rightarrow S_2 \Rightarrow S_3 \Rightarrow S_4$, is compatible with concepts on the step-

ping of the net charge of the catalytic center. Transients of the net charge have been inferred from electrochromic absorption transients in the visible spectral region [5,6]. When starting from the most reduced state, S_0, the relative extent of these absorption transients oscillates as $0:1:0:-1$, which is compatible with a net charge of 0, 0, 1, 1 for states S_0, S_1, S_2, S_3, respectively [7]. This notion has been corroborated by experiments on the rate of reduction of P_{680}^+ by Y_Z. The half-rise times in nanoseconds oscillate as $26:26:50/260:50/260$. The slowing down has been attributed to the Coulombic force exerted by the net charge of the catalytic center before the initiation of the respective transition [8]. The above pattern of net charges was compatible with the then accepted pattern of proton release, namely $1:0:1:2$ for transitions $S_0 \Rightarrow S_1 \Rightarrow S_2 \Rightarrow S_3 \Rightarrow S_4 \to S_0$ [9–12], if it was assumed that the net charge was governed by the difference between electron abstraction and proton release. However, in the pioneering studies on core particles from *Synechococcus* [5–8], proton release has not been assayed in parallel with electrochromism and with the reduction of P_{680}^+. In a more recent study on BBY membranes from spinach, proton release and electrochromism have been compared directly [13]. The pattern of proton release has been shown to be non-integer- and pH-dependent, and the pattern of electrochromic transients seemed to be altered accordingly, again in agreement with the above notion. The first indication for a deviation comes from studies on core particles from spinach and pea where the oscillation of the reduction rate of P_{680}^+ is the same as previously [14], but oscillations of the extent of proton release are absent [15].

In this study we compared both the extent and the kinetics of proton release, electrochromism and electron transfer in thylakoids and core particles. Only these materials are suitable for high time resolution of proton release [2]. We found no correlation between the net charge as based on proton release and the extent of the electrochromic transients. In addition proton release was faster (under certain conditions) than any component of the electrochromic transients.

*Corresponding author. Fax: (49) (541) 969-2870.
Internet: Junge@sfb.biologie.uni-osnabrueck.de

2. Materials and methods

The preparations started with 12-day-old pea seedlings. Unstacked thylakoids were prepared according to [16]. PSII core particles were isolated according to [17]. They were stored frozen at $-80°C$, thawed and suspended at 20 μM (thylakoids) and 3.6 μM (core particles) of chlorophyll for the measurements. With the latter material all media contained 0.01% w/v β-dodecyl maltoside.

Flash-spectrophotometric measurements were performed with the set-up described in [18]. For measurements with dark-adapted material (15 min) the sample was kept in a light-shielded storing vessel. A new charge was filled automatically into the cuvette after each measurement. A Xenon flash (10 μs FWHM) filtered through a long-pass cut-off filter (Schott, RG610) or a Q-switched Ruby-laser (60 ns FWHM) at 694 nm served as exciting light. For signal-to-noise improvement, up to 600 transients were averaged on a Nicolet Pro30 recorder and stored on a MicroVax system.

Electrochromic absorption transients were recorded at a wavelength of 443 nm with dark adapted material in either of two ways: (i) at low time resolution with 1 ms time per address in response to a single group of 9 saturating Xenon flashes of light. The superimposed electrochromic bandshifts due to Q_A^- formation are non-oscillating under the given electron acceptor conditions (see below). They were recorded under repetitive excitation (repetition period: 5 s thylakoids, 0.2 s core particles) and then subtracted from the former ones (see [3]). With thylakoids the media contained 2.6 g/l BSA, with core particles 10 mM MES as pH buffer. (ii) Time resolved absorption transients were recorded at 5 μs per address. They are documented only for the third flash in a series. The Xenon lamp provided two conditioning flashes and the Ruby laser the flash during the recording interval. With thylakoids the media contained no $CaCl_2$. Electrochromism was recorded at 443 nm (no differences between two wavelengths) and electron transfer (Mn to Y_Z^+) at 360 nm [19].

Proton release into the lumen of thylakoids was monitored by absorption transients of the pH-indicating dye Neutral red at 548 nm in the presence of the non-permeable buffer BSA (2.6 g/l) as previously

[20]. DNP-INT (10 μM) blocked proton release at the cytochrome b_6/f protein complex [21]. Proton release by core particles was recorded with the dye Bromocresol purple (30 μM) at 575 nm without added buffer. From transients obtained with the dyes transients measured under the same conditions without dyes were subtracted ($\pm$dye). Proton release was recorded at two different time resolutions (see above). With core particles at 360 nm 100 μM DCBQ, at 443 nm 100 μM DCBQ plus 200 μM FeCy, and at 575 nm 200 μM FeCy were used as electron acceptors. With thylakoids the same acceptor conditions were used at low time resolution and 1 mM FeCy plus 50 μM DCBQ at high time resolution.

The S-state distribution of centers in the dark was evaluated by measuring absorption transients at 295 nm as function of flash number [22] (50 μs per address, optical pathlength 1 cm, see Fig. 1A). All media contained 3 mM $CaCl_2$, 10 mM NaCl, 10 mM MES and 100 μM DCBQ. The portion of each transient which decayed in milliseconds as characteristic for the transition $S_3 \Rightarrow S_4 \rightarrow S_0$ (1.2 ms in thylakoids and 5 ms in core particles) was determined from an exponential fit. The resulting patterns were analyzed, as usual, in terms of the three Kok-parameters: the proportion of photochemical misses, α, of double hits, β, and the percentage of centers in state S_1.

The chlorophyll to reaction center ratio was determined by two procedures: (i) the amount of oxygen released after 200 repetitive Xenon flashes (spacing 100 ms) was measured with a Teflon-covered Clark-type electrode with 200 μM DCBQ plus 400 μM FeCy as electron acceptors. The calibration in terms of mol O_2 per mol chl. was based on the consumption of all disolved oxygen (0.255 mM at 20°C) in the reaction volume (4 ml) which was caused by the addition of FMN (1 mM) under white light illumination. From the calibrated extents and the chlorophyll concentrations we calculated the number of chlorophylls per reaction center. (ii) The formation of Q_A^- was assayed by absorption transients at 320 nm again under repetitive Xenon flash excitation (not shown) with 100 μM DCBQ. The extent at about 50 μs after the flash and using the differential extinction coefficient of the redox couple Q_A^-/Q_A of $\Delta\varepsilon_{320nm} = 1.3 \times 10^4$ $M_{RC}^{-1} \cdot cm^{-1}$ [23] yielded the molar ratio of stable charge separations per chl. The media contained 3 mM $CaCl_2$ plus 10 mM MES, pH 6.5, in both experiments.
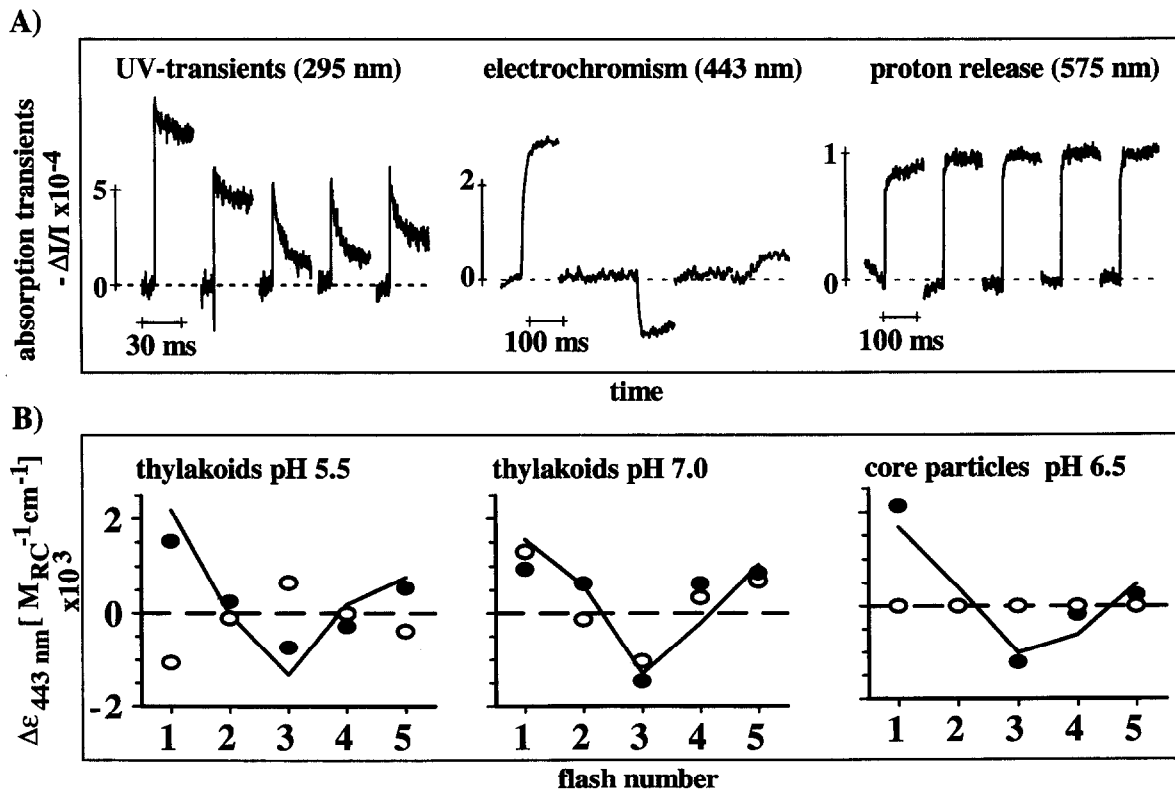

Fig. 1. (A) Original absorption transients at 295 nm (left), 443 nm (dark adapted minus repetitive flash excitation) and of Bromocresol purple at 575 nm induced by the first 5 flashes in PSII core particles. (B) Calibrated extents of stable absorption transients at 443 nm in thylakoids (pH 5.5, left; pH 7.0, middle) and core particles (pH 6.5, right). Experimental data: solid circles, calculated figures based on different assumptions (see text): open circles and lines. The electrical bandwidth was 100 Hz, optical pathlength 2 cm.

3. Results

The chlorophyll-to-reaction center ratios were determined in thylakoids and core particles as described above. We obtained the following figures (UV/O₂): thylakoids 540/550; core particles 130/120. The first value was in agreement with the literature [24] whereas the one for the core particles from pea was about three times higher than originally reported for this type of preparation from spinach [17]. Otherwise the protein composition was similar (of the extrinsic proteins only the 33 kDa was present).

The following Kok parameters were determined from transients at 295 nm (see Fig. 1A, left; section 2) ($\alpha/\beta/S_1$): thylakoids, pH 5.5, 8.0/14.7/93.5; pH 7.0, 2.0/11.1/65.0; and core particles, pH 6.5, 18.0/1.0/91.5. In all cases the centers were well synchronized in the dark. The amount of misses was higher in core particles than in thylakoids correlated well with the reduced antenna size of the former.

The extents of electrochromic transients and proton release were measured as function of flash number in thylakoids and core particles. The transients obtained with core particles at pH 6.5 as induced by the first 5 of a train of nine flashes are shown in Fig. 1A. The extent of the pH-indicating transients was almost constant between flashes (right) whereas the electrochromic transients oscillated. A large positive electrochromic transient on the first flash was almost compensated by a negative transient on the third (middle). Similar experiments were performed with thylakoids (data not documented). The pattern of the pH transient oscillated and differed depending on the pH. At pH 5.5 the extent on the first flash was larger than the

one on the third whereas at pH 7.0 this was reversed, as documented elsewhere [3,4]. The patterns of the electrochromic transients, on the other hand, were similar at both pH values and similar to the ones obtained with core particles. The experimentally obtained extents of transients at 443 nm (see Fig. 1A) were calibrated in terms of the differential molar extinction per reaction center using the above determined antenna sizes. The calculated values are plotted in Fig. 1B as filled circles. The other elements in Fig. 1B (open circles and lines) were calculated under two different sets of assumptions: (i) it was assumed that the relative electrochromic transients behaved as published for core particles from *Synechococcus*, namely as 0:1:0:−1, independent of the pattern of proton release (lines). (ii) Alternatively, it was assumed that the pattern of electrochromic transients strictly followed the pattern of proton release (open circles). In both calculations we used the respective independently determined Kok triples (see above) and the average value of $\Delta\varepsilon(S_1 \Rightarrow S_2)_{443nm} = 2.2 \times 10^3 \ M_{RC}^{-1} \cdot cm^{-1}$ from the literature [25–27]. The comparison of the experimental points (filled circles) with the calculated behaviour (open circles and lines) clearly showed that the pattern of electrochromism was independent of the pattern of proton release and coincident with the previously published pattern in *Synechococcus* [5,6]. It should be noted that we obtained similar results when using BBY membranes or core particles prepared according to another protocol (here not documented).

Comparison of the rates of transient electrochromism, electron transfer and proton release. A straightforward interpretation of time resolved measurements of electrochromism at 443 nm is hampered by the superimposition of absorption
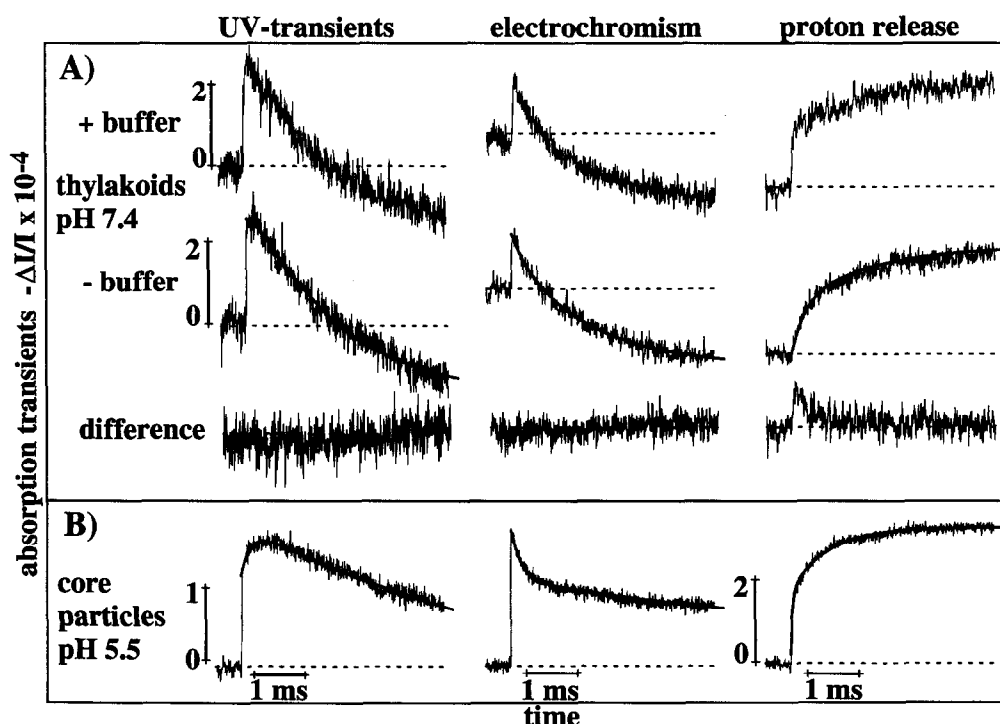

Fig. 2. Absorption transients induced by the third flash in thylakoids (A) and core particles (B). (A) Thylakoids: electron transfer (left, 360 nm) and electrochromism (middle, 443 nm) were recorded in the presence (top row) and absence (middle row) of 10 mM imidazole plus 20 μM Neutral red. Proton release (right) was measured with 20 μM Neutral red (at 548 nm) with (top) and without (middle) 650 μM imidazole. The transient with imidazole was normalized to the same extent of the one without (factor of 2). The differences of transients with/without Neutral red/imidazole are shown in the bottom row. (B) Core particles: transients at 360 nm (left), at 443 nm (middle) and of Bromocresol purple at 575 nm (right). Electrical bandwidth 100 kHz, optical pathlength 1 cm.

transients of different origin, namely electrochromism in response to a new positive charge on manganese or Y_Z, electrochromism in response to Q_A^- and some residual slow absorption transients of P_{680}^+ (see [7]). To overcome this complication we used the variability of the rate of proton release as a kinetic label for those events which are coupled to proton release. The rate of proton release can be speeded up (i) in thylakoids by increasing the concentration of Neutral red or of imidazole, both reacting as mobile buffers by direct collision with the proton donating groups, and (ii) in core particles at acid pH where the deprotonation is due to protolysis and is rapid due to the dominance of groups with a more acid pK (see [2]).

Electron transfer, electrochromism and proton release were time resolved at pH 7.4 in thylakoids and pH 5.5 in core particles on the first four flashes in dark adapted material. Only the third flash is shown in Fig. 2. The half-rise times of the transients were obtained from fits with one or two exponentials (smooth lines).

In thylakoids, all three observables were recorded in the presence of 20 μM Neutral red (Fig. 2A, upper row). Proton release was recorded under addition of 650 μM imidazole and thereby further accelerated (see [4]). Electron transfer and electrochromism were recorded after adding even more, namely 10 mM imidazole, which fully and rapidly quenched all pH-indicating transients of Neutral red and further accelerated proton release. At 360 nm (left) a fast and unresolved jump, mainly due to Q_A reduction [19], was induced by the third flash. It was followed by a decay with half-rise time of 1.1 ms. A small rise with half-rise time of 260 μs was superimposed on the decay. At 443 nm (middle) again a fast jump was apparent which has been previously attributed to the rapid formation of Q_A^- and Y_Z^+, both of which also give rise to local electrochromic shifts [24,28]. It was followed by a biphasic decay with half-rise times (and relative extents) of 250 μs (0.2) and 1.1 ms (0.8). The pH-indicating transient (right) was also biphasic. We observed rapid proton release with a half-rise time of 28 μs (and relative extent of 0.6) and again a ms-phase with half-rise time of 1.1 ms (0.4). The pattern of proton release in the first four flashes proceded as about 0.5:1:1.5:1 as previously reported [16]. We again recorded all three variables, this time in the absence of imidazole and, for electron transfer and electrochromism, also in the absence of Neutral red (Fig. 2A, middle row). The fast phase of proton release (right) on the third flash was much slower, its half-rise time was only 150 μs, whereas the slow phase was unchanged. The half-rise times of electron transfer and electrochromism, on the other hand, were similar to the above figures (lines). The differences (lower row of Fig. 2A) between the transients with (upper row) and without mobile buffers (middle row) further illustrate that the UV and electrochromic transients were almost identical under both conditions. Only the pH transient was significantly accelerated by imidazole. With imidazole the half-rise time of proton release during all other transitions was also 28 μs whereas electron transfer proceded with half-rise times of 37, 74 and 217 μs (start S_0). We determined the deuterium/hydrogen isotope effect on the third flash. For electron transfer and electrochromism the ratio was about 1.3 for proton release about 3.

With core particles (Fig. 2B) proton release was recorded with 50 μM Bromocresol purple without further buffers, electron transfer and electrochromism in the presence of only 50 μM MES. At 360 nm the jump on the third flash (left) was

followed by a small rise with half-rise time of 150 μs and a larger decay with half-rise time of 6 ms. The electrochromic transient at 443 nm (middle) decayed with half-rise times of 140 μs (0.35) and 6 ms (0.65). Proton release (right) was fast and biphasic with half-rise times of 28 μs (0.55) and 520 μs (0.45), respectively. The same half-rise times were obtained on the other transitions whereas the ones of electron transfer were 81, 76 and 204 μs (start S_0), respectively.

Summarizing, the results of the time resolved measurements on the third flash in both types of materials: the major portion of proton release was much faster than electron transfer from Mn to Y_Z^+. The electrochromic transients in both materials decayed in milliseconds concomittant with electron transfer. A minor μs-phase ($t_{1/2} \approx 200$ μs) was still slower than the fast component of proton release ($t_{1/2} \approx 30$ μs). It might be attributable to centers in states other than S_3 prior to the third flash (about 20% in thylakoids and 35% in core particles) or to contributions from the acceptor side. Whereas fast proton release in thylakoids was accelerated by imidazole, electron transfer and electrochromism were constant.

4. Discussion

When the oxygen evolving system is clocked through its oxidation states the pattern of proton release greatly varies as function of the material and the pH, and the rate varies as a function of the pH in core particles and of the concentration of mobile, amphiphilic buffers in thylakoids [2]. These variations are not paralled by variations of the pattern of oxygen evolution and of the UV transients, which are indicative of manganese redox transitions [3]. In this work we investigated the pattern of electrochromic transients which might reflect the net charge transients of the catalytic center as the result of electron abstraction and proton release. We found the pattern of electrochromic transients hardly affected by variations of the extents and, even more meaningful, the rates of proton release. These observations are in line with those of van Leeuwen [28] that there are pronounced oscillations of the rate of the reduction of P_{680}^+ in core particles although oscillations of proton release are absent.

Because of their spectral features the electrochromic origin of certain absorption transients in the blue (chlorophyll), the green (carotenoid) and the red (chlorophyll) region is beyond doubt [5,26,29]; and the original interpretation is plausible, i.e. that Coulombic force exerted by a positive surplus charge in the catalytic center slows the electron transfer from Y_Z to P_{680}^+ [7]. It is also plausible that the transients of electrochromism and of the rate of P_{680}^+ reduction are indicative of internal charge displacements; as opposed to the net charge resulting from electron abstraction and to proton release into the medium.

The term net charge has to be qualified. If the newly deposited positive charge (e.g. on manganese) is more deeply immersed in the protein than a given proton releasing group at the protein/electrolyte boundary, the electrostatic compensation of the newly created positive charge by the release of one proton would be incomplete. The results of this work revealed that the electrochromic probes are positioned close to (or more favourably oriented towards) manganese and Y_Z and far away from (or unfavourably oriented towards) the sites of proton release. This supports the notion [2,4] that the observable proton release is a peripheral phenomenon.

During each of three transitions (except in thylakoids for the oxygen evolving $S_3 \Rightarrow S_4 \rightarrow S_0$ [4]) we observed the same extent of proton release at 10 μs time resolution and in milliseconds. In other words the deprotonation was the same no matter whether the positive charge resided on Y_Z or on the manganese cluster. Moreover, we found no influence of the deprotonation pattern on the rates of the electron transfer between manganese and Y_Z^+. This suggests that the system [manganese–Y_Z] may be located in a pocket with high dielectric strength, which is not unlikely considering the polar and ionic ligands to manganese and the close vicinity of ionic cofactors like Cl^- and Ca^{2+} [1].

The chlorophyll *a* molecules which are responsible for electrochromism in the blue are more closely apposed to Y_Z than to the manganese cluster. This results from time resolved experiments ([30,31], see also [2] and this work). The stable electrochromic band shift upon transition $S_1 \Rightarrow S_2$, that is only reversed upon the oxygen evolving transition, $S_3 \Rightarrow S_4 \rightarrow S_0$, is then indicative of the deposition of one positive charge in the manganese center. The absence of a bandshift during transition $S_2 \Rightarrow S_3$, on the other hand, then implies (i) the perfect compensation of the newly deposited positive charge e.g. by an intra-protein proton shift or, alternatively, (ii) the placement of this electron hole on a cofactor which is farther apart from the electrochromically responsive pigments. Further insight is expected from ongoing studies on the spectra of electrochromism and from the application of electrometric techniques.

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