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Evidence for a chemical reaction of hydroxylamine with the photosynthetic water splitting enzyme S in the dark – possible states of manganese and water in the S cycle

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Measurement of oxygen yield, UV- and electrochromic absorption changes were made with isolated oxygen-evolving PS II complexes of the cyanobacterium *Synechococcus* sp. After treatment with low concentrations of hydroxylamine the corresponding flash patterns showed a delay of two S-state transitions of the water-splitting enzyme S compared to untreated samples. The delay is still observed 10 h after removal of unreacted hydroxylamine by gel filtration. This indicates a chemical reaction of hydroxylamine with the water-splitting enzyme in the dark. The consequences for the possible states of manganese (as carrier of redox equivalents) and of water in the quarternary S cycle are discussed.

Introduction

In oxygen-evolving PS II, a light-driven one-electron transport chain is coupled to the water oxidizing complex, S, which stores four oxidizing equivalents prior to the oxidation of 2 H_2O to 1 O_2. In the primary act after light excitation a stable, transmembrane charge separation [1] takes place between a special chlorophyll *a*, Chl-a_{II} (P680) [2,3], and a special plastoquinone, Q_A [4]. Subsequently Chl-a_{II}^+ oxidizes a tyrosine [5–7], which in turn extracts an electron out of the enzyme S.

By consecutive extraction of four electrons, the enzyme S undergoes a change between the states S_0 to S_4. Oxygen is released upon transition from S_4 to S_0 [8,9]. It is generally assumed that 4 Mn are engaged in this reaction. At least two of them may be redox-active.

Abbreviations: Chl, chlorophyll; DCBQ, 2,5-dichloro-*p*-benzoquinone; β-DM, β-dodecyl D-maltoside; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; HMCM, 40 mM Hepes-Na/0.3 M mannitol/20 mM $CaCl_2$/10 mM $MgCl_2$; Mes: 2-(*N*-morpholino)ethanesulfonic acid; MMCM, as HMCM, only 20 mM Mes instead of Hepes; OEC, oxygen-evolving complex; PPBQ, phenyl-*p*-benzoquinone; PS II, Photosystem II; P700, reaction center of Photosystem I; SB 12, sulfobetaine 12; SiMo, silicomolybdate.

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Electrochromic absorption changes around 690 nm observed with S-state transitions indicate the creation of a surplus charge in S_2 and S_3 [10]. This can be explained if, together with the transitions $S_0 \rightarrow S_1 \rightarrow S_2 \rightarrow S_3 \xrightarrow{(S_4)} S_0$ and electron extraction 1:1:1:1 in the inner sphere, an H^+ release takes place that follows a pattern of 1:0:1:2. The charges on S_2 and S_3 also explain the retardation of the electron transfer to Chl-a_{II}^+ [11]. The 1:0:1:2 H^+ release is identical with that observed in the outer water phase by pH indicators [12,13]. This is by no means self-evident, since pH indicators located outside the membrane may detect H^+ dissociation from proteins at the periphery, later replaced by protons from water. This is in line with recent results [14,15] which show that the external proton release pattern is strongly influenced by changes in the environment of the water-splitting enzyme. This is not the case with the electrochromic absorption changes (see Discussion). Therefore, the H^+ stoichiometry concluded from the electrochromic changes in the inner sphere may indicate the true pattern of proton release from water.

Absorption changes in the UV have been attributed to changes of the oxidation state of manganese in PS II [16–18]. There is now an agreement that the difference spectrum for the $S_0 \rightarrow S_1$ transition is different from that of the $S_1 \rightarrow S_2$ and $S_2 \rightarrow S_3$ transitions; the latter two are similar [19,20]. The spectrum for the $S_0 \rightarrow S_1$

transition in Ref. 20 is, however, not identical with that reported in Refs. 18, 19. Based on the analysis of our UV absorption changes we have proposed [18] that two manganese within a cluster of four Mn's change their redox state in the S-cycle and have the following possible oxidation states: S_0(Mn(II), Mn(III)); S_1(Mn(III), Mn(III)); S_2(Mn(III), Mn(IV)) and S_3(Mn(IV), Mn (IV)).

In the dark S_1 is the only stable state [21] and the sequence of S-state transitions starts with $S_1 \rightarrow S_2 \rightarrow S_3 \xrightarrow{(S_4)} S_0$. Treatment of S_1 with low concentrations of the reducing agent hydroxylamine leads to a delay in the S-state transitions for two states, so that five flashes instead of three are required before oxygen is evolved [22]. The delay can be observed in the pattern of UV and electrochromic absorption changes as well [17]. It is obvious that in the presence of NH_2OH the transitions are $S_x \rightarrow S_0 \rightarrow S_1 \rightarrow S_2 \rightarrow S_3 \xrightarrow{(S_4)} S_0$. S_x is a new state, supplemented by additional absorption changes with the transition $S_x \rightarrow S_0$.

The different manganese valence states may be available if for one of the S states the Mn-valences can be estimated. We have given arguments that the S_x state is favourable for such a calibration [18] (see Discussion). Therefore, the nature of S_x is of interest. It is still a matter of debate:

(1) whether NH_2OH shifts the S_1 state backwards to S_x already in the dark by a chemical reaction with S_1 and which oxidation state of Mn is present in S_x;
(2) whether NH_2OH is merely reversibly bound to the S_1 state in the dark; i.e., $S_x = (S_1 \cdot n\ NH_2OH)$, so that a backward shift occurs only after transition of $S_1 \cdot n\ NH_2OH$ to $S_2 \cdot n\ NH_2OH$ in the first flash and a subsequent rapid reduction of the latter to S_0.

In this work we have performed measurements of oxygen yield, UV and electrochromic absorption changes of samples treated with NH_2OH and after removal of the unreacted hydroxylamine, to clarify whether in the dark a chemical reaction or a reversible binding of NH_2OH occurs. A short communication of the following results has already been presented at the VIIIth International Congress on Photosynthesis in Stockholm (1989) [23].

Materials and Methods

Measurements of oxygen yield were performed as described in Ref. 24. Absorption changes at two different wavelengths were measured simultaneously over cross with a double-beam spectrometer for each wavelength as described in Ref. 25. The light source in the UV was a high-pressure mercury lamp (Original Hanau, St 75) and in the red spectral range a Quartz-Halogen lamp (Osram). The light path through the solution (5 μM Chl) was 1 cm and in the red 5 cm. The saturating laser flashes ($t_{1/2} = 300$ ns) from a Phase-R dye laser (dye: Rhodamine 6G) were spaced at 500 ms.

O_2-evolving PS II complexes from the cyanobacterium *Synechococcus* sp. have been prepared according to Refs. 26 and 27. They were stored at −80°C in 20 mM Mes-Na (pH 6.5), 20 mM $CaCl_2$, 10 mM $MgCl_2$, 1 M sucrose and 0.06% SB 12.

For the measurements, the PS II stock solutions were diluted with media containing 40 mM Hepes-Na (pH 7) or 20 mM MES-Na (pH 7), 0.3 mannitol, 20 mM $CaCl_2$ and 10 mM $MgCl_2$ (HMCM and MMCM, respectively). The preparations were dark-adapted for at least 30 min after thawing. In some experiments 0.03% β-DM was added. Stock solutions with hydroxylamine were freshly prepared from hydroxylammonium chloride (E. Merck). After addition of 25 μM NH_2OH to the samples they were incubated for 10–20 min at room temperature.

PD-10 gel-filtration columns (Pharmacia) with Sephadex G 25 M were used to remove NH_2OH after incubation. These gel-filtration columns can lower the concentration of small molecules and ions by a factor of at least 500, as was checked for the case of chloride and calcium with ion-selective electrodes. If samples are treated with hydroxylamine (25 μM), the activity of O_2 evolution declines by 70% in 10 h, but the activity immediately after gel filtration of such samples is the same as that measured 10 h later. This indicates that this method is useful for a powerful removal of

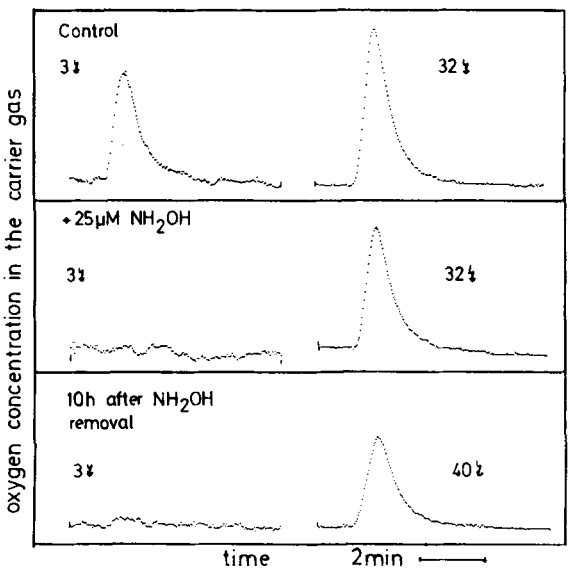

Fig. 1. Oxygen yield under different conditions after 3 and 32–40 flashes (1 Hz): Top: dark-adapted samples; Center: after 10 min incubation with 25 μM NH_2OH (HMCM, pH 7); Bottom: after removal of NH_2OH by gel filtration (MMCM, pH 6). The samples (volume 1 ml) contained 30 μM Chl, 200 μM PPBQ, 0.03% β-DM. The signals (different scales) correspond to 0.216 nmol O_2 (top, left), 2.90 nmol (top, right), 2.31 nmol (center, right), 1.81 nmol (bottom, right).

NH_2OH. The columns were equilibrated with media containing MMCM (pH 6 or 7). Further details are described in the text and in the figure legends.

Results

The extent to which the oxygen evolution pattern was shifted by hydroxylamine was investigated by measuring the oxygen yield of dark adapted samples illuminated with three flashes. A lack of oxygen evolution could either be due to a shift or to a deactivation caused by the different treatments described. Therefore, about 10 min later, the oxygen yield after 32–40 flashes was measured with the same samples to check the activity. In Fig. 1 the signals due to oxygen evolution after three flashes are shown on the left side, whereas on the right side the signals after 32–40 flashes are depicted.

Samples that have not been treated with hydroxylamine evolve 7 mmol O_2/mol Chl in three flashes and 97 mmol O_2/mol Chl in 32 flashes (Fig. 1, top). If the samples have been incubated with 25 μM hydroxylamine for about 10 min, no oxygen is evolved in three flashes, whereas 80% of the control yield (untreated sample) is evolved in 32 flashes (Fig. 1, center). Therefore, the water oxidation activity has only slightly decreased and the absence of oxygen evolution after three flashes indicates the expected delay of the S-state transitions through NH_2OH.

If, after 10 min incubation, hydroxylamine is efficiently removed by gel filtration (see Material and Methods), the delay can still be observed (Fig. 1, bottom). This is in agreement with results by Bouges, who dialyzed NH_2OH-treated samples against NH_2OH-free buffer [22].

Since, after removal of unreacted NH_2OH, eventually bound hydroxylamine might only slowly dissociate into the surrounding medium, we waited up to 10 h before measuring oxygen yields. Furthermore, we changed the pH from 7 during the incubation to 6, which was the pH of the medium with which the gel-filtration column had been equilibrated. This pH change should further reduce the concentration of the free base form of hydroxylamine, which was reported to be the active species [28]. However, neither condition lead to a reappearance of the usual O_2-evolution pattern (Fig. 1, bottom). The loss of activity compared to the untreated sample is due to the reduced stability of NH_2OH-treated samples.

Fig. 2, top left, shows the pattern of the absorption changes in the UV (367 nm) without NH_2OH-treatment. On the first two flashes an absorption increase can be seen, which corresponds mainly to the $S_1 \rightarrow S_2$ and $S_2 \rightarrow S_3$ transitions, respectively. The absorption increase is reversed upon the third flash, when S_3 goes to S_0 (via S_4), together with oxygen release. As a fraction of the centers does not make a turnover upon a flash (misses), whilst others make a double turnover

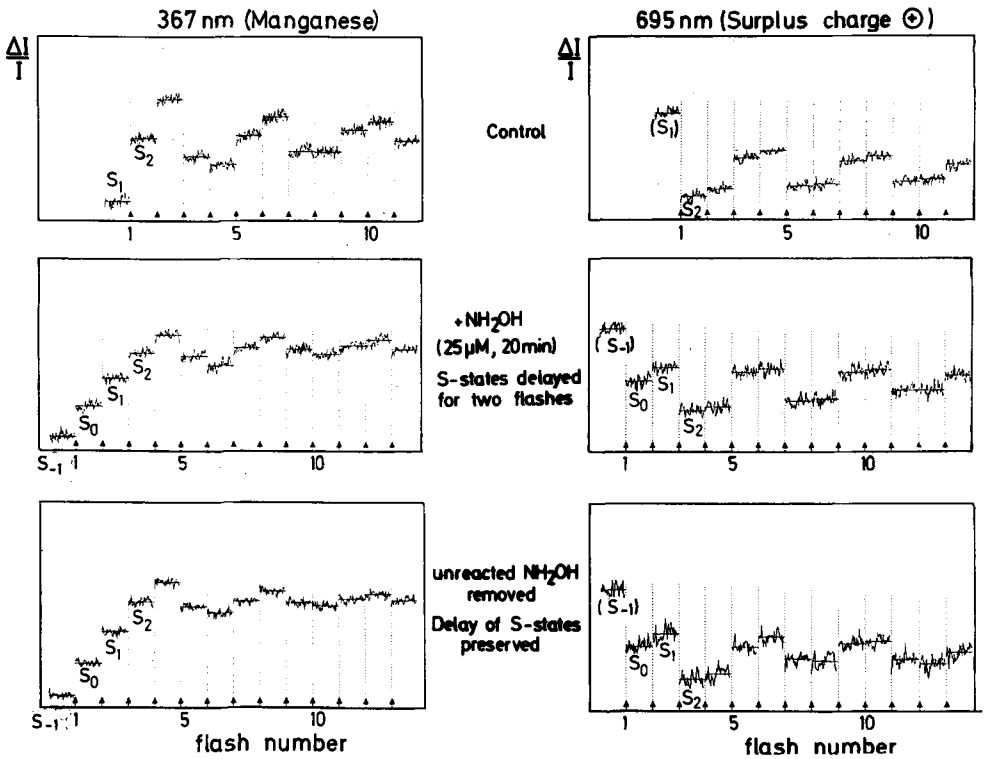

Fig. 2. Left: UV-absorption changes at 367 nm; right: electrochromic absorption changes at 695 nm. Top: untreated sample; center: incubated with 25 μM NH_2OH for 20 min; bottom: 3 h after NH_2OH-removal by gel filtration. The samples contained MMCM (pH 7), 5 μM chl, 5 μM SiMo (acceptor added 3 min before the measurements).

(double hits), the S-state-transitions become more and more mixed the higher the flash number. Therefore, the oscillation pattern is damped.

The pattern of electrochromic absorption changes at 695 nm is shown in Fig. 2, top right, for an untreated sample. There are negative absorption changes on the 1st, 5th and 9th flash and positive absorption changes on the 3rd, 7th and 11th flash. The negative absorption changes are due to the creation of a positive surplus charge with the $S_1 \rightarrow S_2$ transition (the first flash also contains solitary contributions from P700 and the acceptor side, indicated by brackets). The charge on S_2 remains on S_3 and disappears with the $S_3 \rightarrow S_0$ transition in the 3rd, 7th, 11th flash, etc., indicated by a positive absorption change for these transitions.

After dark incubation with 25 μM hydroxylamine for 20 min, we obtain the UV and electrochromic absorption changes shown in the center of Fig. 2 (left and right), which were measured simultaneously. For both wavelengths the characteristics of the pattern after the second flash are the same as those of the pattern of untreated samples; i.e., a delay of two S-state transitions has taken place and mainly S_1 is reached after two flashes.

As shown for the O_2 evolution, after removal of NH_2OH by gel filtration the delay is still present (Fig. 1, bottom). A two-state delay after NH_2OH removal is observable also in the case of the absorption changes at 367 and 695 nm (Fig. 2, bottom, left and right).

As mentioned above, misses and double hits lead to a damping of the oscillation patterns. Values for the amount of misses and double hits as well as of the initial S-state distribution can be obtained by fitting the oscillation patterns with these parameters as well as with the $\Delta\epsilon$ values at 367 nm used in Ref. 18. For the UV pattern shown in Fig. 2, top, this results in 70% of the centers intially in S_1, 30% in S_0 (or in S_1 with reduced donor D), 8% misses and 10% double hits. The relatively high amount of double hits is caused by the measuring beam at 695 nm. After hydroxylamine treatment, 95% of the centers are initially in S_x, but while the double hits remain practically constant (9%), the misses have doubled (16%). This is due to the interaction of hydroxylamine with the higher S-states between the flashes [29]. After removal of NH_2OH the miss parameter is reduced to 10% (with 93% S_x, 10% double hits).

We did not find evidence that the reaction of NH_2OH with the water-splitting enzyme in the dark is influenced by chloride, as outlined by Beck and Brudvig [28], who observed an inverse dependence of the reaction rate on the chloride concentration. With an excess of Cl^- (MMCM + 300 mM NaCl) we could still observe the two-state delay after dark incubation with 25 μM hydroxylamine for 20 min, as shown in Fig. 3. Fitting of the UV-absorption changes gave 98% S_1,

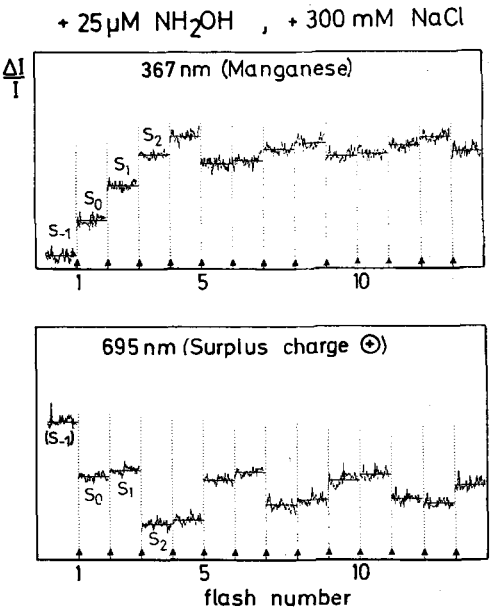

Fig. 3. Top: UV-absorption changes at 367 nm; bottom: electrochromic absorption changes at 695 nm. The sample was incubated with 25 μM NH_2OH for 20 min and contained MMCM (pH 7), 300 mM NaCl, 5 μM Chl, 600 μM DCBQ (acceptor added 1 min before the measurement).

18% misses and 12% double-hits. The same results were obtained for Cl^- concentrations down to 3 mM (not shown).

Discussion

The delay of the patterns of O_2 evolution, UV and electrochromic absorption changes remains for hours after removal of NH_2OH by gel filtration. This indicates that a chemical reaction in the dark has taken place prior to the removal, resulting in a new reduced state, i.e., $S_x = S_{-1}$. It is designated S_{-1} because it is delayed from S_1 for two states (see above). For the same reason, S_{-1} must be formed by reduction of S_1 through two electrons from hydroxylamine.

A very-high-affinity binding of NH_2OH to S_1 as an alternative explanation cannot be strictly excluded solely on the basis of the preserved delay. However, the pattern of the absorption changes in the UV cannot be explained by a binding of NH_2OH to S_1: Namely, if S_{-1} is $S_1 \cdot n \cdot NH_2OH$ (see above), this means that the large absorption difference between S_1 and S_{-1} (see Fig. 2, left, center and bottom) must be caused by the binding of NH_2OH, which is very unlikely.

As a chemical reaction of NH_2OH with S_1, a reduction of manganese is probable [18,30]. This should end in the lowest Mn valence value, Mn(II). To explain the two-flash delay, S_{-1} must be two electrons more reduced than S_1 (see above). This can be realized if S_{-1} contains at least two Mn(II) and S_1 two Mn(III) within

the cluster. Based on this calibration, the S-states should contain in respect to the redox active manganese: S_0 (Mn(II), Mn(III)), S_1 (Mn(III), Mn(III)), S_2 (Mn(III), Mn(IV)) and S_3 (Mn(IV), Mn(IV)). In S_4 oxidized Tyr may represent the 4th oxidizing equivalent, i.e., S_4 (2Mn(IV), Tyr^+).

An explanation for a two-electron difference is also possible if, for example S_1 is (Mn(II), Mn(IV)). Mn(IV) in S_1 can, however, be excluded, because S_1 is the only stable state in the dark; whereas, Mn(IV) is not a stable state at physiological conditions. The absence of Mn(IV) in S_1 was also shown by measurements of the relaxation rates for NMR signals of protons [31] and the EPR signal of tyrosine-D^+ [32] as well as measurements of magnetic susceptibility of the S-states [33].

These oxidation states of the redox active manganese in the different S-states are in agreement with the fact that the UV-absorption changes of the $S_0 \rightarrow S_1$ transition are different from those of the $S_1 \rightarrow S_2$ and $S_2 \rightarrow S_3$ transitions, which are similar (see Introduction). The former would be a Mn(II) → Mn(III) change, while the latter two would both be an Mn(III) → Mn(IV) change. The S_{-1} state outlined above as S_{-1}(2 Mn(II)) would also explain the additional absorption change in the UV upon the $S_{-1} \rightarrow S_0$ transition (Fig. 2, left).

One could consider that not only the two redox active Mn's in S_1 are reduced in the dark from Mn(III) to Mn(II) by NH_2OH, but also the additional manganese in the cluster, if they are in the state Mn(III). This was recently observed through a three-flash delay after long NH_2OH incubation times (hours instead of minutes). This delay became evident through an additional step of UV as well as electrochromic absorption changes below S_{-1}, indicating the formation of an S_{-2} state. Therefore, in the cluster still a further less accessible Mn(III) ion may exist which is reduced in the $S_{-1} \rightarrow S_{-2}$ transition. This additional Mn(III) might be oxidized by Tyr^+ in S_4, i.e., S_4(3Mn(IV)), see above (Kretschmann and Witt, unpublished data).

Our above assignment of the Mn-valences to the different S states is in agreement with X-ray absorption edge measurements of Mn with regard to the $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_2$ transitions [34], as both are accompanied by a shift of the edge to higher energy, indicating an oxidation of manganese. Furthermore, the multi-line EPR signal of Mn in S_2 [35] is in accordance with Mn(III/MnIV) in S_2 However, an Mn edge shift is observed neither after addition of NH_2OH in the dark nor for the $S_2 \rightarrow S_3$ transitions [34,36]. An edge shift to lower energy, indicating a reduction of manganese, occurs only upon illumination of NH_2OH-treated samples. These results may be reconciled with our proposed model of Mn-oxidation states if one considers that redox active amino acids (AC) ligate manganese or are at least close to it (tyrosine, for example, is an effective electron carrier between Mn and Chl-a_{II} [5–7]).

Other associated amino acids (AC) (or other, unidentified components) could be in a redox equilibrium with manganese: S_{-1}(Mn(II), Mn(II), AC[O]) ↔ S_{-1}(Mn(III), Mn(III), AC(−II)). This equilibrium can be shifted to the right at low temperatures and to the left at room temperatures. Such a shift of a redox equilibrium has been observed by EPR of a molybdenum-containing enzyme [37].

In the X-ray absorption experiment at 170 K a manganese reduction would therefore not be observed, in contrast to the UV-absorption experiment at room temperature. The observed lowering of the X-ray absorption edge energy after illumination of S_{-1} can also be explained by this hypothesis, as (Mn(IV), Mn(III), AC(−II) formed after the illumination might be unstable and transform into S_0(Mn(II), Mn(III), AC [O]).

Likewise the absence of an X-ray edge shift for the $S_2 \rightarrow S_3$ transition might be due to an oxidation at low temperatures of, for instance, a histidine by Mn(IV) in S_3 whereby Mn(IV) reverts to Mn(III). Such an electron exchange between manganese and histidine may also occur at other abnormal conditions, e.g., through Ca^{2+} depletion [38]. The UV-difference spectrum of the $S_2 \rightarrow S_3$ transition under normal conditions without Ca^{2+} depletion at room temperature is not compatible with that observed in Ref. 39 for a histidine oxidation.

Apart from manganese, the S states also contain, probably as ligands of manganese, water and its derivatives, respectively. For an estimation of these states of water, we again consider the S_{-1} state as basis. After the first flash and the electron extraction in the $S_{-1} \rightarrow S_0$ transition, the release of 2 H^+ was observed [40]. This was also concluded from the appearance of a negative surplus charge measured through electrochromism at $S_{-1} \rightarrow S_0$ [17]. As NH_2OH reduces the S_1 state to S_{-1} and is thereby oxidized itself, the two protons released in the $S_1 \rightarrow S_0$ transition upon the first flash cannot originate from NH_2OH but only from water. Since 4 H^+ are released in the sequence $S_{-1} \rightarrow S_3$ (2 H^+ with $S_{-1} \rightarrow S_0$, 1 H^+ with $S_0 \rightarrow S_1$, 1 H^+ with $S_2 \rightarrow S_3$), consequently two neutral H_2O are located in S_{-1}, i.e., it is S_0(2 OH^-) (see also Ref. 41). The other states then have to be $S_1(OH^-, O^{2-})$, $S_2(OH^-, O^{2-})$ and $S_3(O^{2-}, O^{2-})$ [18].

As has been shown, the delay in the S-state transitions caused by hydroxylamine can be observed in the patterns of oxygen evolution, UV and electrochromic absorption changes equally well. Apart from the delay, i.e., the appearance of the S_{-1} and S_0 state through reduction of S_1 by NH_2OH, the structure of the UV and electrochromic patterns of the S_1 and further S-states has not changed through the treatment with NH_2OH. This is understandable if the two optical patterns reflect properties directly related to the states

of manganese and water within the quaternary S-cycle. This also means that the H^+ stoichiometry concluded from the electrochromic absorption changes might indicate the proton releases from the water in the active site.

In contrast to this, measurements of proton release by pH-indicators may not be directly related to the protons from water. This is indicated by the following results. With PS II reaction centre complexes from pea, no delay was observed after hydroxylamine treatment, nor was the pattern affected by Cl^--depletion, although in both cases the oxygen evolution pattern was changed [14]. Similarly, preparations of PS II core complexes from spinach did not even show a period-four oscillation in the proton release pattern when measured with pH-indicators [15].

The discrepancy with the results in Ref. 28 with regard to the influence of chloride is probably due to the different preparations used. It was shown that the 17 and 23 kDa polypeptides of higher plant PS II specifically affect and modify the reaction between hydroxylamine and the S-state enzyme [42]. The Cl^- concentration required for water oxidation also depends on the presence of these two polypeptides. It is therefore possible that they are the cause for the effect of chloride on the reaction with hydroxylamine.

In our experiments we used PS II preparations from a cyanobacterium, which lack the 17 and 23 kDa polypeptides and have no specific requirement for chloride [43]. Thus there is no evidence that a common binding site plays a role in the reaction of hydroxylamine with the water-splitting enzyme.

The reduction of S_1 through NH_2OH as a basis of the conclusions outlined in this work will be supported in a forthcoming paper. There, the oxidation of NH_2OH in the dark is shown directly by mass spectroscopic detection of the oxidation products.

Acknowledgements

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