Biochimica et Biophysica Acta 850 (1986) 184–196
Elsevier

BBA 42010

Studies on the nature of the water-oxidizing enzyme. III. Spectral characterization of the intermediary redox states in the water-oxidizing enzyme system Y

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(Received December 23rd, 1985)

Key words: Photosystem II; Water splitting; Redox state; Redox center; S-state transition

Spectral changes in the ultraviolet region (250–380 mn) that are caused by redox transitions within the water-oxidizing enzyme system Y were analyzed by measuring time-resolved (1–2 μs) absorption changes in dark-adapted PS II membrane fragments. In order to eliminate effects due to the interference with binary oscillation at the acceptor side the experiments were performed in trypsinized (at pH = 6.0) samples with 0.4 mM $K_3[Fe(CN)_6]$ as exogenous oxidant. In the presence of 10 mM $CaCl_2$ PS II-membrane fragments trypsinized at pH = 6.0 are fully competent in oxygen evolution compared with the control (measured as average oxygen yield per flash). It was found: (1) Excitation of dark-adapted samples with a train of saturating laser flashes (7 ns full width at half maximum, FWHM) induces absorption changes with resolved kinetics in the micro- and millisecond range. Except for the kinetically unresolved initial amplitude the other transient components are strongly dependent (in extent and half-life time) on the flash number. (2) After elimination of the oxygen-evolving capacity by 1 mM hydroxylamine (NH_2OH) binary oscillation are completely lacking of absorption changes at 325 nm. The remarkably smaller absorption change in the first flash can be explained by partial oxidation of acceptor 'C400' due to $K_3[Fe(CN)_6]$. (3) A significantly different pattern of the absorption changes is observed if phenyl-*p*-benzoquinone is used instead of $K_3[Fe(CN)_6]$. Based on the oscillations of the initial amplitude of the absorption changes, exogenous quinones are assumed to give rise to a more complicated reaction pattern. (4) The spectral analysis performed on the basis of Kok's model (Kok, B., Forbush, B. and McGloin, P.M. (1970) Photochem. Photobiol. 11, 457–475) by the use of α, β and the apparent $[S_0]/[S_1]$ ratio experimentally determined via oxygen-yield measurement leads to the separation of the difference spectra $\Delta\varepsilon(S_i \rightarrow S_{i+1})$ for the redox transitions in system Y. The obtained difference spectra are almost identical for the $S_0 \rightarrow S_1$ and $S_2 \rightarrow S_3$ transitions, while for $S_1 \rightarrow S_2$ a markedly different spectrum has been observed. The deviations are especially pronounced in the range around 325 nm. (5) The relaxation kinetics of absorption changes at 325 nm that are characterized by a 1 ms half-life time oscillate in their amplitude synchronously with the oxygen yield in a flash train. The amplitude of these kinetics as a function of wavelength corrected for the Z^{ox}/Z difference spectrum closely resembles the calculated spectrum for the $S_3 \rightarrow S_0$ transition. Based on the experimental data a model is proposed for the molecular mechanism of photosynthetic water oxidation that is an extension of our previous hypothesis (Renger, G. (1977) FEBS Lett. 81, 223–228). Accordingly, the catalytic site for water oxidation is assumed to be a binuclear manganese center which undergoes redox reactions at the manganese centers only during $S_1 \rightarrow S_2$ and $S_3 \rightarrow S_0$ transitions. The implications of this model are discussed.

Abbreviations: Ph-*p*-BQ, phenyl-*p*-benzoquinone; Mes, 4-morpholineethanesulfonic acid.

Introduction

Photosynthetic water oxidation to molecular dioxygen takes place via a four-step univalent redox-reaction sequence that is driven by a photooxidized chlorophyll-*a* complex, referred to as P-680$^+$. The dark reactions leading from H_2O to O_2 are performed within a manganese-containing protein designated as water-oxidizing enzyme system Y (for a review, see Ref. 1). The understanding of the molecular mechanism requires a detailed knowledge of the nature of the intermediary redox states in system Y and of the dynamics of processes that give rise to the redox transitions $S_i \rightarrow S_{i+1}$. Here the index i denotes the number of oxidizing redox equivalents stored in system Y.

One way to attack this problem is the attempt of a spectral characterization of the S_i states. During the last years progress has been achieved in labeling especially the S_2-state by its low temperature EPR-signal that exhibits a characteristic multiline hyperfine structure [2–4]. A theoretical analysis of the spectra led to the conclusion that the signal arises from a binuclear manganese center in a mixed valence state which magnetically couples with another spin-1-state metal center [5]. Information about the EPR-properties of intermediary redox states other than S_2 are almost completely lacking. The electronic spectra of the S_i states are unknown. So far only attempts are known to identify the difference spectra of the $S_i \rightarrow S_{i+1}$ redox transitions. All spectroscopic studies are based on the characteristic damped oscillation pattern in the S_i-state population that arises after excitation of dark-adapted samples with a train of single turnover flashes. This S_i-state 'labeling', however, becomes complicated in the case of the electronic spectra because of the overlapping of absorption changes caused by PS II acceptor side reactions which exhibit a damped binary oscillation pattern due to the gating mechanism of plastoquinol formation (for a review, see Ref. 6). Accordingly, the crucial point of all spectral analysis of the $S_i \rightarrow S_{i+1}$ redox transitions is the elimination of this gating mechanism or a proper account for the effects due to these binary oscillations. The first attempt in this direction has been reported by Velthuys [7] who used incubation with hydroxylamine (NH_2OH) to eliminate the oxygen-evolving capacity. Based on the assumption that NH_2OH does not affect the gating mechanism at the acceptor side, the absorption change differences between control and NH_2OH-treated samples were used to determine the difference spectra for $S_1 \rightarrow S_2$ and $S_3 \rightarrow (S_4) \rightarrow S_0$ [7]. A completely different approach was used by Renger and Weiss [8,9]. We eliminated binary oscillations at the acceptor side by a mild trypsination which is known to interrupt the electron transport between Q_A and Q_B and to permit a sufficiently fast Q_A^- reoxidation with $K_3[Fe(CN)_6]$ as exogenous electron acceptor [10,11]. It was found that under these circumstances absorption changes induced by a flash train in trypsinized thylakoids reveal a 1 ms kinetics which oscillate in their amplitudes synchronously with the oxygen yield [8,9]. The difference spectrum with a peak around 325 nm was inferred to reflect the redox reaction $Z^{ox}S_3 + 2H_2O \rightarrow ZS_0 + O_2 + 2H^+$ [12]. A more appropriate sample material became available with the development of procedures that permitted the isolation of PS II membrane fragments, which are highly active in O_2 evolution [13]. This material has been used to separate the difference spectra for all redox transitions $S_i \rightarrow S_{i+1}$ [14]. However, it was concluded that the spectra obtained have to be corrected for contributions due to binary oscillations at the acceptor side. Based on this assumption all $S_i \rightarrow S_{i+1}$ transitions ($i = 0 \ldots 3$) were inferred to be characterized by the same difference spectrum. The shape of this spectrum was claimed to indicate that every redox step in system Y implies an Mn(III) $\rightarrow$ Mn(IV) transition. As a consequence of this analysis the binary oscillation of the acceptor side was assumed to be correlated with the S_i-state transitions. As this assumption has very important implications for the mechanism of water oxidation, an alternative approach was used in the present study in order to test the above mentioned idea. In the present study again the trypsination method using $K_3[Fe(CN)_6]$ as exogenous oxidant was applied in order to eliminate binary oscillations due to the acceptor side. The results obtained do not support the above-mentioned conclusion. It is concluded that the difference spectra of $S_0 \rightarrow S_1$ and $S_2 \rightarrow S_3$ transitions are very similar, whereas the spectrum for $S_1 \rightarrow S_2$ is quite different. The mechanistic consequences of these findings are discussed.

Materials and Methods

All experiments including the trypsin treatment were performed at pH = 6.0. The standard suspension contained: PS-II-membrane fragments trypsinized at pH = 6.0 (10 μM chlorophyll)/10 mM NaCl/10 mM $CaCl_2$/0.4 mM $K_3[Fe(CN)_6]$/20 mM Mes-NaOH, pH = 6.0. Other additions as indicated in the figure legends.

The measurements of the oscillation pattern of oxygen yield performed by a Joliot-type electrode [16] as well as the numerical parameter fit were outlined in Ref. 17. In order to separate the difference spectra for the different redox transitions ($S_i \rightarrow S_{i+1}$) a matrix analysis has been used that is based on the Kok-model [18] and on parameters α, β and $[S_0]/[S_1]$ obtained from the oxygen-yield measurements. Absorption changes were measured as described in Ref. 15. The optical band width was 4 nm.

In order to prove that the trypsinized samples were fully competent in oxygen evolution in the presence of $CaCl_2$ the average oxygen yield was measured by the conventional method with a Clark-type electrode [19,20].

Results

Kinetical pattern of absorption changes in dark-adapted trypsinized PS II membrane fragments

All methods used for the analysis of intermediary redox states of the water-oxidizing enzyme system rely on their characteristic oscillation pattern with a periodicity of 4 that is observed after illumination of dark-adapted samples with a train of single turnover flashes [21,22]. The analysis of absorption changes measured under these conditions becomes complicated by contributions with a periodicity-2 oscillation that arises due to the gating mechanism for plastoquinol formation at the acceptor side of PS II (for a review, see Ref. 6). Accordingly it seems worthwhile to simplify the system by eliminating these binary oscillations. For intact thylakoids this has been shown to be achievable by mild trypsination and use of $K_3[Fe(CN)_6]$ as terminal electron acceptor [10,11]. Under these conditions the electron transport between the primary and secondary plastoquinone acceptors, Q_A and Q_B, is blocked and Q_A^- becomes directly reoxidized by $K_3[Fe(CN)_6]$. In the absence of $K_3[Fe(CN)_6]$ the Q_A^- reoxidation by S_2 exhibits the same kinetics as in normal thylakoids in the presence of DCMU [8]. It appears reasonable to use this trypsination method for PS-II membrane fragments also. Measurements of the average oxygen yield per flash revealed that trypsination at pH = 6.0 does not affect the number of functionally competent water-oxidizing enzyme systems Y, provided that 10 mM $CaCl_2$ is present in the measuring suspension (Völker, M., Eckert, H.J. and Renger, G., unpublished results). Absorption changes induced at 360 nm by a train of laser flashes in dark-adapted trypsinized (pH = 6.0) PS II membrane fragments are shown in Fig.

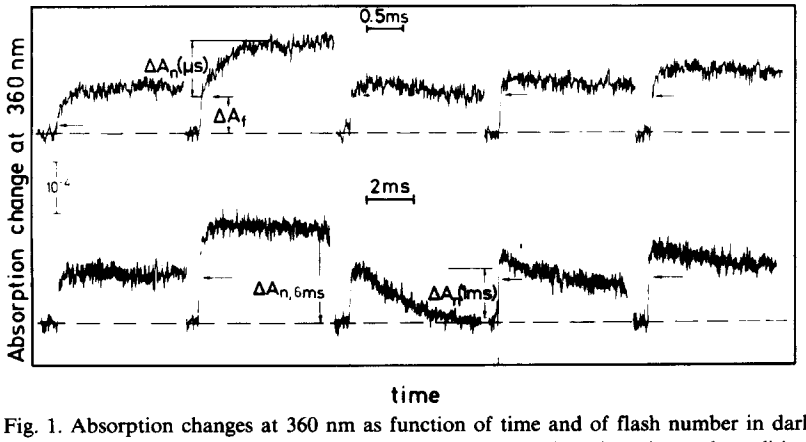


Fig. 1. Absorption changes at 360 nm as function of time and of flash number in dark-adapted trypsinized (pH = 6.0) PS-II-membrane fragments excited with a train of saturating laser flashes. Experimental conditions as described in Materials and Methods.

1. These traces exhibit four kinetically distinguishable contributions. (a) Fast rise that is limited by the time resolution of the equipment (1–2 μs). Except for the 1st flash, the extent of this contribution, ΔA_f, is practically independent of the flash number in the sequence. (b) Rise kinetics in the μs range. The extent of this contribution, ΔA_n (μs), markedly depends on the flash number. It reflects redox transitions in system Y. (c) Relaxation kinetics with $t_{1/2} = 1.0–1.3$ ms. The extent of this contribution, ΔA_n (1 ms) exhibits a characteristic oscillation during the flash sequence. (d) Amplitude still remaining 5 ms after the flash. The extent of this absorption, $\Delta A_{n,5\,ms}$, also strongly depends on the flash number n.

The ΔA_f data have been used in the previous study for the determination of the Z^{ox}/Z-difference spectrum [15]. Here we will analyse the components that are related to the intermediary redox states of system Y. At a first glance, most of the information is obtained from the $\Delta A_{n,5\,ms}$ values. 5 ms after flash excitation the transient electron-transfer steps in system Y are almost completely accomplished, whereas effects due to the deactivation of S_2 and S_3 are negligibly small [22–24]. Therefore, $\Delta A_{n,5\,ms}$ should reflect mainly the redox state of system Y plus contributions due to the acceptor side. If binary oscillations at the acceptor side do not occur in mildly trypsinized samples, the contributions due to these reactions should be the same in each flash. The acceptor-side contribution can be calculated as the average value of $\Delta A_{n,5\,ms}$ for the n flashes in the sequence because under steady-state conditions the net contribution of the $S_i \rightarrow S_{i+1}$ state transitions in system Y vanishes at 5 ms. Before going into the details of the calculation, the crucial point of the whole analysis first remains to be checked; i.e., whether or not binary oscillations take place at the acceptor side under our experimental conditions.

Analysis of acceptor side contributions to absorption changes in dark adapted trypsinized PS II membrane fragments

To eliminate oscillating absorption changes due to the reaction sequence in system Y experiments have been performed in samples that were completely deprived of their oxygen-evolving capacity by incubation with 1 mM NH_2OH. Absorption changes were measured at 325 nm, the positive maximum for Q_A^--formation. The obtained data depicted in Fig. 2 show that binary oscillations of $\Delta A_{n,5\,ms}$ are totally lacking. This result unfortunately does not provide an unambiguous proof for the absence of binary oscillations in trypsinized PS-II particles in the absence of NH_2OH because this compound could be responsible for the Q_A–Q_B disconnection rather than the trypsin treatment (at pH = 6.0) itself. However, for the following reasons the later possibility seems to be unlikely:

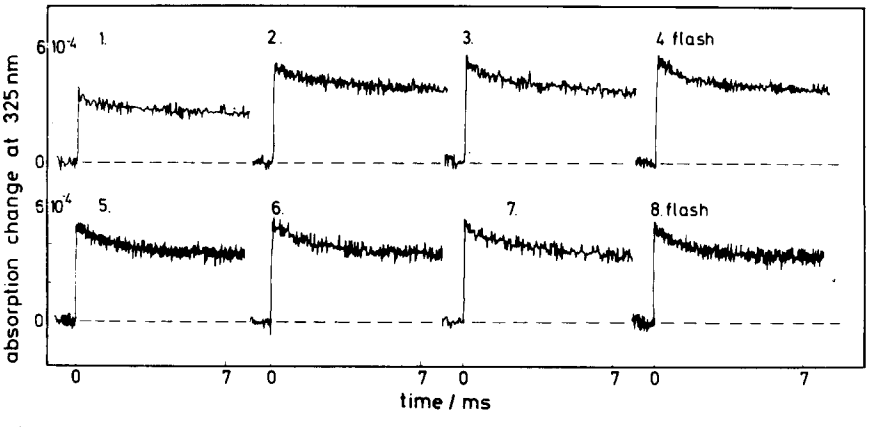


Fig. 2. Absorption changes at 325 nm as a function of time and of flash number in dark-adapted trypsinized (pH = 6.0) PS-II-membrane fragments excited with a train of saturating laser flashes. The oxygen-evolving capacity was completely eliminated by incubation with 1 mM NH_2OH. Other experimental conditions as described in Material and Methods, except for using 15 μM chlorophyll instead of 10 μM.

(a) in normal thylakoids the binary oscillation due to the gating mechanism at the acceptor side remains unaffected by 1 mM NH_2OH [25,26] and (b) trypsination of PS II membrane fragments at pH = 6.0 reduces herbicide binding (Renger, G., Hagemann, R., and Fromme, R., unpublished results). This indicates a significant functional change of the acceptor side that should markedly diminish the stability of Q_B^-, thereby eliminating the binary oscillation pattern at the acceptor side. A striking phenomenon of Fig. 2 is the remarkably smaller amplitude of absorption changes due to the first flash. A comparable effect was also observed for ΔA_f in Fig. 1. In order to analyze the origin of this phenomenon, ΔA_f as a function of wavelength was depicted for the values obtained in the 1st flash and compared with the average values taken from ΔA_f of the 2nd, 3rd and 4th flash. The data are shown in Fig. 3.

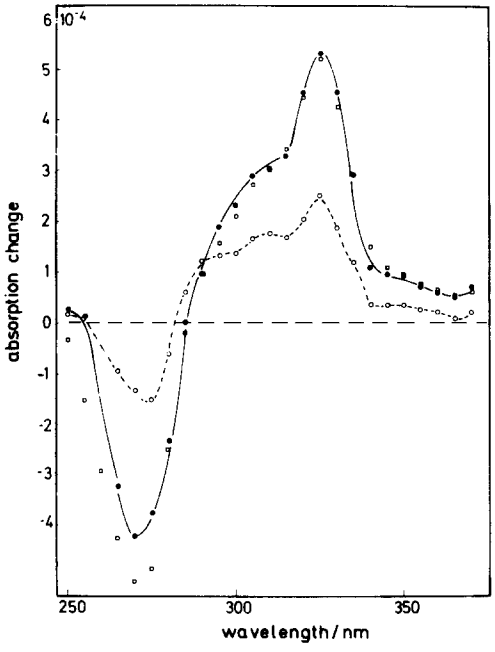


Fig. 3. Extent of the kinetically unresolved 'initial' amplitude as a function of wavelength in dark-adapted trypsinized (pH = 6.0) PS-II-membrane fragments excited with a train of saturating laser flashes. ○, 'initial' amplitude of absorption changes due to the 1st flash; ●, average 'initial' amplitude obtained from the values due to the 2nd, 3rd and 4th flash; □, 'initial' amplitude of the 1st flash plus a fraction of the Q_A^-/Q_A-difference spectrum (weighing factor, 0.63). Other experimental conditions are as described in Materials and Methods.

Remarkable deviations are observed in the ranges of 265 nm and of 325 nm. After addition of a Q_A^-/Q_A difference spectrum with a weighing factor of 0.63 the spectral differences almost completely disappear. This suggests that the origin of the decreased absorption changes in the 1st flash could be due to a diminished 'detected' Q_A^- formation. A likely explanation for this phenomenon is the assumption that $K_3[Fe(CN)_6]$ oxidizes the acceptor component 'C400' [27,28] and that the fast electron transfer from Q_A^- to $C400^{ox}$ prevents its detection due to our limited time resolution (1–2 μs) for the data of Figs. 2 and 3. This assumption would additionally imply that the difference spectrum for $C400^{ox}/C400^{red}$ is rather

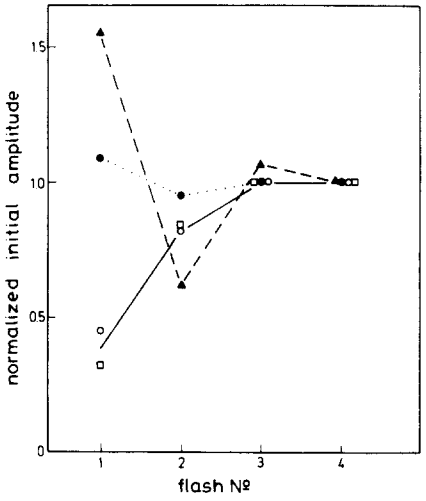


Fig. 4. 'Initial' amplitude of absorption changes at 320 nm as a function of flash number in dark-adapted PS II-membrane fragments excited with a train of saturating laser flashes. It has to be emphasized that in this case the assay conditions differed from those described in Materials and Methods. The reaction mixture contained: PS-II-membrane fragments (10 μM chlorophyll), 10 mM NaCl and 20 mM Mes-NaOH, pH = 6.2. Further additions or sample treatment are as noted below. ○, control samples in the presence of 0.2 mM $K_3[Fe(CN)_6]$; the pattern remains unaffected by additions of 10 mM $CaCl_2$ or by mild trypsinations at pH = 6.0. Likewise, addition of 0.01 up to 1 mM NH_2OH to trypsinized PS II membrane fragments in the presence of 10 mM $CaCl_2$ does not change the pattern; □, Tris-washed PS II membrane fragments in the presence of 0.2 mM $K_3[Fe(CN)_6]$; ■, control samples in the presence of 0.2 mM $K_3[Fe(CN)_6]$ and 1 mM NH_2OH, but absence of $CaCl_2$; ▲, control samples in the presence of 0.1 mM Ph-*p*-BQ; mild trypsination at pH = 6.0 and addition of 10 mM $CaCl_2$ does not modify the pattern.

small in the range of 250–380 nm. Highly time-resolved measurements are required to clarify this point. If the smaller initial amplitude of 325 nm absorption is due to a 'C400'-type effect, the phenomenon should strongly depend on the nature of the exogenous acceptor, but should be independent of the functional integrity of the water-oxidizing enzyme system Y, provided that appropriate donors are present. The data obtained under various experimental conditions are summarized in Fig. 4. It is confirmed that destruction of system Y by Tris-washing or NH_2OH addition does not markedly change the extent of the $K_3[Fe(CN)_6]$-induced decrease of ΔA_f at 325 nm in the 1st flash. If NH_2OH is added without $CaCl_2$, the effect practically disappears. This dependence of the NH_2OH-action on $CaCl_2$ remains to be clarified. Fig. 4 suggests that the decrease of the initial amplitude in the 1st flash is an acceptor side effect. If this is really the case then the extent of absorption changes reflecting the turnover of P-680 should remain constant. Absorption changes induced by a laser flash train in dark-adapted trypsinized (pH = 6.0) PS-II-membrane fragments after addition of NH_2OH and $CaCl_2$ are shown in Fig. 5. Virtually no oscillation of the initial amplitude is observed, while under the same conditions ΔA_f at 325 nm induced by the 1st flash is less than 50% of the ΔA_f in the following flashes (see Fig. 4). Therefore, these data support the idea of a $K_3[Fe(CN)_6]$-induced 'C400'-effect. A surprising result, however, was obtained in the presence of

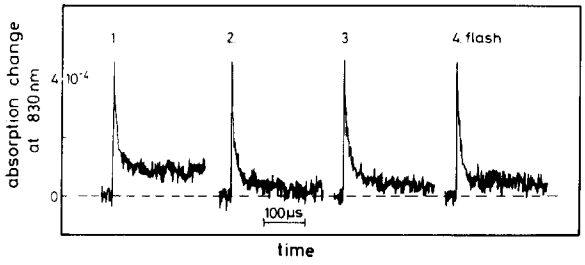


Fig. 5. Absorption changes at 830 nm as a function of time and of the flash number in dark adapted trypsinized (pH = 6.0) PS-II-membrane fragments excited with a train of laser flashes. The oxygen evolving capacity was completely eliminated by addition of 1 mM NH_2OH. Other experimental conditions as described in Materials and Methods, except for using 30 µM chlorophyll.

the lipophilic acceptor compound phenyl-*p*-benzoquinone (Ph-*p*-BQ). In this case, a remarkable binary oscillation with a high damping is observed for ΔA_f at 325 nm (see Fig. 4). As the time resolution of these measurements for the detection of ΔA_f was better than 5 µs the normal Q_A–Q_B gating mechanism with electron-transfer times of 150–200 µs ($Q_A^- Q_B \rightarrow Q_A Q_B^-$) and 450 µs ($Q_A^- Q_B^- \rightarrow Q_A Q_B^{2-}$) [29,30] cannot account for this oscillation. Excitation with 7 ns laser flashes also eliminates the possibility of double-hit effects. The oscillations of Fig. 4 appear to be related to the acceptor side, because after Tris-washing the pattern was not changed basically, only the damping slightly increased (data not shown). Regardless of the unresolved mechanism it is clear that Ph-*p*-BQ introduces additional effects that could influence the separation of absorption changes due to the redox transitions in system Y. Therefore, for the following analysis only $K_3[Fe(CN)_6]$ was used as exogeneous electron acceptor.

Determination of difference-extinction coefficients $\Delta\varepsilon_{i,i+1}(\lambda)$ from amplitudes $\Delta A_{n,\,5ms}(\lambda)$

Typical traces of absorption changes induced at 360 nm by a train of laser flashes in dark-adapted trypsinized (pH = 6.0) PS-II-membrane fragments in the presence of 0.4 mM $K_3[Fe(CN)_6]$ and 10 mM $CaCl_2$ were shown in Fig. 1. If one accepts that under these conditions binary oscillations due to the acceptor side are completely eliminated, the absorption change at 5 ms after the nth laser flash is given by:

$$\Delta A_{n,5\,ms}(\lambda) = \left[\sum_{i=0}^{3}\{(1-\alpha)[S_i]_{n-1} + \beta[S_{i-1}]_{n-1}\}\Delta\varepsilon_{i+1,i}(\lambda)\right] c_Y d + \Delta A_{5ms}^{acc}(\lambda) \quad (1)$$

In Eqn. 1 $[S_i]_{n-1}$ describes the probability that the redox state S_i ($i = 0,\ldots,3$) is populated after the $(n-1)$th flash in system Y; c_Y represents the total concentration of system Y; $\Delta\varepsilon_{i+1,i}(\lambda)$ are the difference extinction coefficients for the redox transition $S_i \rightarrow S_{i+1}$ in system Y; d is the optical pathlength and $\Delta A_{5ms}^{acc}(\lambda)$ represents the contribution due to the acceptor side that is assumed to be constant in each flash (except for the 1st flash,

vide supra). Based upon the above-mentioned considerations $\Delta A^{acc}_{5\,ms}(\lambda)$ has been calculated as the average value of $\Delta A_{n,\,5\,ms}(\lambda)$ for $n = 2$–8. Theoretical calculations based on the Kok model [18] show that under these conditions the contributions due to the donor side is negligigbly small (data not shown). The data obtained are depicted in Fig. 6. A comparison with the Q_A^-/Q_A difference spectrum taken from the literature [31] readily shows that $\Delta A^{acc}_{5\,ms}(\lambda)$ cannot be simply explained by the assumption of Q_A^- reoxidation with $K_3[Fe(CN)_6]$ being the only contribution. Correction factors for particle flattening are small in PS II membrane fragments between 250 nm and 380 nm (the variations are less than 5%, see Ref. 31). Therefore, this effect is negligibly small (it is within the experimental error of the data).

One might speculate about the possibility of plastoquinol formation if one assumes that in trypsinized PS-II-membrane fragments plastoquinone can accept electrons from Q_A^- and subsequently undergoes dismutation. This mechanistic point, however, would be relevant for the following analysis only if the reaction causes an oscillation of $\Delta A^{acc}_{5\,ms}(\lambda)$ within the flash sequence. There is no evidence for such a phenomenon (vide supra). Hence, without considering the details of the $\Delta A^{acc}_{5\,ms}$ spectrum depicted in Fig. 6, these values were used in order to correct the $\Delta A_{n,\,5\,ms}(\lambda)$ values for the constant acceptor side contributions. The data obtained for $\Delta A_{n,\,5\,ms}(\lambda)$ are depicted in Fig. 7. From these spectra $\Delta\varepsilon_{i+1,\,i}(\lambda)$ can be calculated by the system of linear equations that arises from a set of four $\Delta A^{corr}_{n,\,5\,ms}(\lambda)$ values ($n = k$, $k+1$, $k+2$, $k+3$) by using Eqn. 1 after subtraction of $\Delta A^{acc}_{5\,ms}(\lambda)$. As in the first flash the initial amplitude was markedly smaller, the $\Delta A_{n,\,5\,ms}(\lambda)$ values for $n = 1$ were not used for our spectral analysis in order to avoid interference with unknown contributions from the acceptor side. Accordingly, $\Delta A^{corr}_{1,\,5\,ms}(\lambda)$ is meaningless and has not been depicted in Fig. 7. The S_i-state population probability as well as α and β were experimentally determined by oxygen-yield measurements with a Joliot-type electrode and numerical parameter fitting as described in Ref. 17. Using these values ($\alpha = 0.20$; $\beta = 0.05$; $[S_0] = 0.15$ and $[S_1] = 0.85$) the spectra depicted in Fig. 8

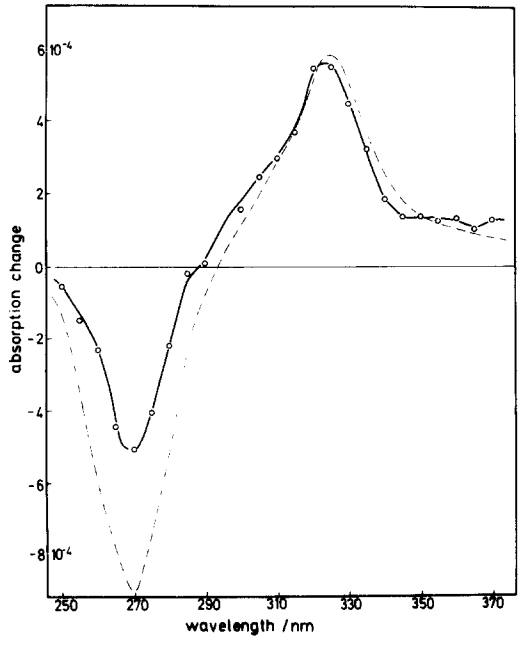


Fig. 6. Average value of the amplitude at 5 ms after excitation of 325 nm absorption changes due to the 2nd–8th flash in dark-adapted trypsinized (pH = 6.0) PS-II-membrane fragments. Experimental conditions as described in Material and Methods. For comparison the Q_A^-/Q_A-difference spectrum is given (dashed curve).

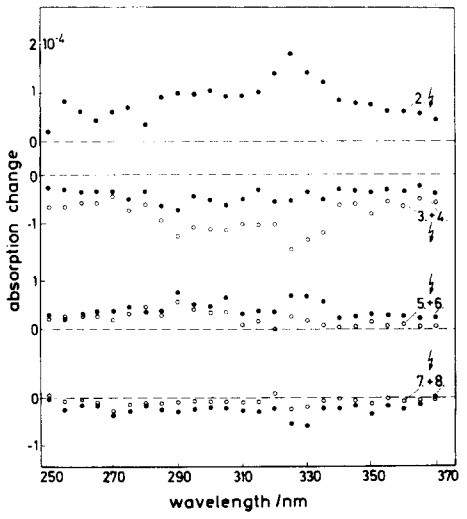


Fig. 7. Difference between the amplitudes at 5 ms after flash excitation and the average values of Fig. 6 as a function of wavelength of absorption changes induced by the 2nd–8th laser flash in dark-adapted trypsinized (pH = 6.0) PS-II-membrane fragments. Experimental conditions as described in Material and Methods.

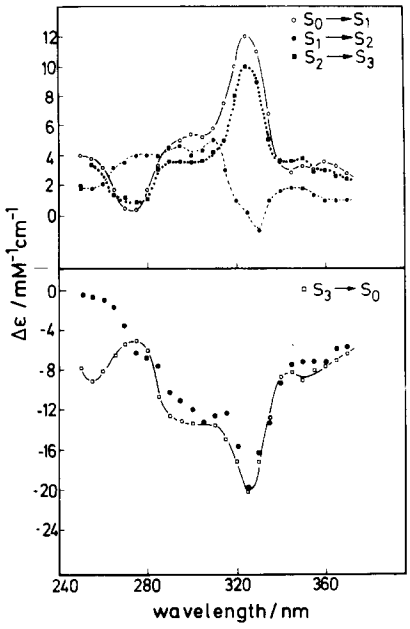


Fig. 8. Difference spectra for the redox transition $S_i \rightarrow S_{i+1}$ obtained from the data in Fig. 7 by using Eqn. 1 for numerical evaluation. Dots in the lower part are obtained by subtracting the difference spectrum of Z^{ox}/Z [15] from the difference spectrum of the 1 ms kinetics.

are obtained. c_Y of Eqn. 1 has been determined from the average oxygen yield per flash measured with a sensitive Clark-type electrode (for details, see Ref. 20).

The data in Fig. 8 exhibit characteristic features. At a first glance the difference spectra for the redox transitions $S_0 \rightarrow S_1$ and $S_2 \rightarrow S_3$ are very similar, whereas the spectrum for $S_1 \rightarrow S_2$ markedly differs. The most pronouncing differences are observed in the range around 325 nm with smaller deviations around 270 nm. This might suggest an interference with binary oscillations due to the PS II acceptor side, as has been recently discussed [14].

However, the present study does not provide any evidence for the existence of binary oscillations caused by the acceptor side in trypsinized PS II membrane fragments with 0.4 mM $K_3[Fe(CN)_6]$ (vide supra). Before going into details of this problem it seems worthwhile to check the possibility of an independent method for the determination of $\Delta\varepsilon_{i+1,i}(\lambda)$.

Determination of $\Delta\varepsilon_{S_3/S_0}$ from the extent of the oscillating 1 ms relaxation kinetics ΔA_n (1 ms)

It has previously been shown, that in dark-adapted trypsinized thylakoids with $K_3[Fe(CN)_6]$ as exogenous acceptor the absorption changes at 325 nm exhibit a multiphasic relaxation with a 1 ms component that oscillates in its extent synchronously with the oxygen yield in a flash train [9,12]. In order to prove that this relation also exists in trypsinized (pH = 6.0) PS-II-membrane fragments, comparative measurements were performed of absorption changes at 325 nm and of the oxygen yield induced by a train of laser flashes in dark-adapted samples. The data obtained are depicted in Fig. 9. They clearly show a very close correspondence in the oscillation pattern. Based on kinetic arguments the 1 ms-relaxation kinetics was inferred to reflect the reaction $Z^{ox}S_3 + 2H_2O \rightarrow ZS_0 + 2H^+ + O_2$ [12]. Accordingly, the extent of ΔA_n (1 ms) as a function of wavelength should represent the sum of the difference spectra $\Delta\varepsilon_{Z^{ox}/Z}$ plus $\Delta\varepsilon_{S_3/S_0}$. On the basis of these considerations $\Delta\varepsilon_{S_3/S_0}$ can be obtained by subtraction of $\Delta\varepsilon_{Z^{ox}/Z}$ given in Fig. 9 of our previous study [15] from the ΔA_n (1 ms) values that are transformed in $\Delta\varepsilon$ units. The results obtained are shown in Fig. 8 as dots. A good correspondence is observed for the comparison of these data with those calculated by the use of the $\Delta A_{n,\,5\,ms}(\lambda)$ values and the numerical analysis according to Eqn. 1. This favors the idea that the spectral analysis outlined in Fig. 8

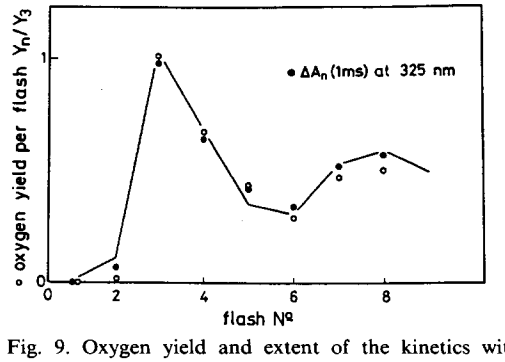


Fig. 9. Oxygen yield and extent of the kinetics with 1 ms half-life time of 325 nm absorption changes as a function of flash number in dark-adapted trypsinized (pH = 6.0) PS-II-membrane fragments excited by a train of saturating laser flashes. Experimental conditions as described in Material and Methods.

really reflects redox transitions within the water oxidizing enzyme system Y rather than reactions at the acceptor side.

Comparison of calculated and measured maxima of absorption changes as a function of flash number

A further test that has been performed in this study is the calculation of the maximum amplitudes of the absorption changes at different wavelengths as a function of flash number. The kinetics of redox transitions in the water-oxidizing enzyme system of mildly trypsinized (pH = 6.0) PS-II-membrane fragments have been determined recently [24]. The following data have been obtained:

$t_{1/2}(S_0 \rightarrow S_1) = 40 \pm 20\ \mu s$; $t_{1/2}(S_1 \rightarrow S_2) = 110 \pm 30\ \mu s$

$t_{1/2}(S_2 \rightarrow S_3) = 220 \pm 40\ \mu s$; $t_{1/2}(S_3 \rightarrow S_0) = 1.2 \pm 0.2$ ms

These results are very similar to recent data obtained in PS II membrane fragments without trypsin treatment [23]. Accordingly, mild trypsination (pH = 6.0) in the presence of $CaCl_2$ does not only maintain the full oxygen-evolving capacity, but also leaves the kinetics of the electron transfer step between the water-oxidizing enzyme system Y and Z^{ox} practically unaffected.

With the kinetical data and by the use of $\Delta\varepsilon_{i+1,i}(\lambda)$ from Fig. 8, of $\Delta\varepsilon_{Z^{ox}/Z}$ from Ref. 15 and of $\Delta\varepsilon_{Q_A^-/Q_A}$ from Ref. 31 the maxima of the absorption changes were calculated with the assumption that Z^{ox} directly reacts with system Y: $Z^{ox}S_i \rightleftharpoons ZS_{i+1}$ (for the sake of simplicity deprotonation steps are omitted). A comparison of the experimental results with the calculated values is depicted in Fig. 10. No data are given for the 1st flash, because of the uncertainty in the degree of $\Delta\varepsilon_{Q_A^-/Q_A}$ contribution. Fig. 10 reveals that the experimental data at different wavelengths can be satisfactorily described.

In summary, the present study shows that within the framework of the underlying model a consistent description can be achieved for the donor-side reactions in trypsinized PS-II particles that are fully competent in their oxygen-evolving capacity.

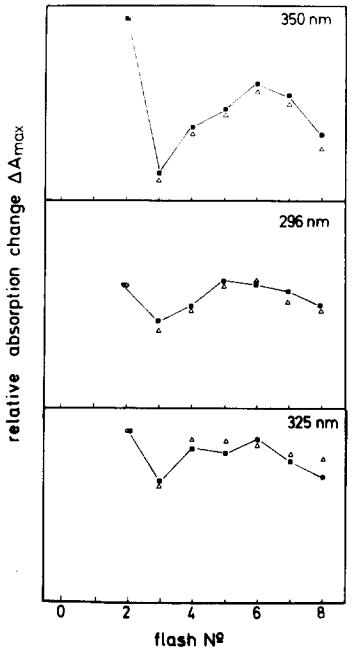


Fig. 10. Maximal amplitudes of absorption changes at 296 nm, 325 nm and 350 nm as a function of flash number in dark-adapted trypsinized (pH = 6.0) PS-II-membrane fragments excited by a train of saturating laser flashes. Experimental conditions as described in Material and and Methods. ■, experimental data; △, calculated values (see text).

Discussion

Interpretation of the spectral data

The spectral analysis presented in this study reveals that the difference spectra of the redox transitions $S_0 \rightarrow S_1$ and $S_2 \rightarrow S_3$ are very similar, whereas the $\Delta\varepsilon_{S_1 \rightarrow S_2}$ spectrum is quite different in the region around 325 nm and to smaller extent around 270 nm. Regardless of the differences in spectral shape a similar phenomenon has also been observed by Dekker et al. [14]. Two basically different interpretations with important implications for the mechanism of water oxidation can be offered. (a) The spectral differences of the calculated $\Delta\varepsilon_{S_i,S_{i+1}}$ values do not reflect the participation of different redox groups in the water-oxidizing enzyme system Y, but are due to the overlapping of absorption changes that are caused by acceptor side reactions which exhibit binary oscillations. This interpretation has been proposed by Dekker et al. [14]. Accordingly, these authors concluded that each redox transition $S_i \rightarrow S_{i+1}$ in the

water-oxidizing enzyme system Y implies the same type of reaction. Based on spectral comparison with model manganese complexes, this reaction was assumed to be an Mn(III) oxidation to Mn(IV) [14]. (b) The spectral differences of the calculated $\Delta\varepsilon_{S_i,S_{i+1}}$ values indicate that really different types of reaction are responsible for the redox transitions $S_i \rightarrow S_{i+1}$. This idea is generally in line with previous models [7,32,33].

At a first glance our data could be described within the first interpretation (data not shown). In this case the differences between the spectra reported by Dekker et al. [14] and our data in Fig. 8 could be simply explained by a shift of the binary oscillation at the acceptor side by one flash. This effect could be due to $K_3[Fe(CN)_6]$-induced C400 oxidation which is slow enough to prevent reoxidation of reduced C400 (formed in the first flash via Q_A^-) during the flash sequence. However, we do not favor the first interpretation for the following reasons: (1) Mild-trypsin treatment of thylakoids interrupts the electron transfer between Q_A and Q_B as effectively as addition of DCMU [8]. It seems unlikely that a similar effect should not arise in PS-II-membrane fragments. (2) Recent data show that trypsination at pH = 6.0 reduces herbicide binding (Renger, G., Fromme, R. and Hagemann, R., unpublished results). Likewise Q_B^- should become destabilized and in the presence of 0.4 mM $K_3[Fe(CN)_6]$ (at 10 μM chlorophyll in the suspension) Q_B^- could hardly survive between the flashes. (3) After destruction of the oxygen-evolving capacity by 1 mM NH_2OH, binary oscillations of $\Delta A_{n,5\,ms}$ are totally lacking (see Fig. 2). One might argue that this effect is caused by NH_2OH. Indeed, it was found that NH_2OH affects herbicide binding in thylakoids [34] as well as in PS II membrane fragments (Renger, G., Fromme, R. and Hagemann, R., unpublished results). However, it appears unlikely that the disappearance of binary oscillations in the latter sample type is due to 1 mM NH_2OH alone rather than due to trypsin treatment because in normal thylakoids NH_2OH does not affect the gating mechansim at the acceptor side [25,26], whereas mild trypsination completely blocks the electron transfer from Q_A^- to Q_B [8].

If one accepts that in trypsinized (pH = 6.0) PS-II-membrane-fragments in the presence of 0.4 mM $K_3[Fe(CN)_6]$ a binary oscillation due to acceptor-side reactions does not exist, then our results indicate that the $S_i \rightarrow S_{i+1}$ redox reactions are not identical processes for $i = 0$, 1 and 2. In order to account for the differences to previous results [14] we have to assume that they are due to different experimental conditions. This idea is supported by our finding that the kinetically unresolved initial amplitudes in the same sample type (PS-II-membrane fragments trypsinized at pH = 6.0, $\pm CaCl_2$) strongly depend on the nature of the exogenous acceptor (see Fig. 4). It is especially interesting to note that in the presence of Ph-*p*-BQ a pronounced and highly damped binary oscillation is observed. This unresolved phenomenon might indicate that exogenous quinones could give rise to additional overlapping effects in the ultraviolet.

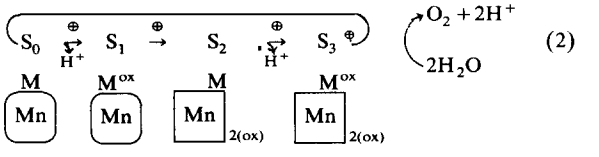
The above-mentioned consideration have serious implications for the molecular mechanisms of photosynthetic water oxidation. If the redox transitions $S_0 \rightarrow S_1$ and $S_2 \rightarrow S_3$ give rise to very similar difference spectra, while $S_1 \rightarrow S_2$ exhibits a markedly different behaviour, it appears reasonable to assume, that $S_0 \rightarrow S_1$ and $S_2 \rightarrow S_3$ reflect the same process and $S_1 \rightarrow S_2$ is basically a different type of reaction. Therefore the question arises about the nature of the redox groups that perform these reactions.

Model for the redox reaction sequence in the water-oxidizing enzyme system

It is well known that manganese is involved in the redox reaction sequence of system Y (for recent reviews, see Refs. 1, 35 and 36). This implies that at least one reaction reflects a redox transition of manganese. Recently, absorption changes in the near-infrared have been discovered that were observed only during the $S_1 \rightarrow S_2$ and $S_3 \rightarrow S_0$ redox steps. These long-wave transitions are ascribed to intervalence electron transfer in a mixed valence manganese cluster [37]. Accordingly, the $S_1 \rightarrow S_2$ transition is assumed to be due to oxidation of manganese in the catalytic site, whereas the reduction takes place during $S_3 \rightarrow S_0$. In order to account for the extremely high stability of the redox state S_1 the existence of a further redox active group M has been postulated [32,33]. M could be either another transition metal center

or a ligand of the manganese-cotnaining catalytic site [1,9,12]. If one combines this idea with the present data it appears reasonable to ascribe the difference spectrum of $S_0 \rightarrow S_1$ of Fig. 8 to the redox transition $M \rightarrow M^{ox}$. This assignment implies that also $S_2 \rightarrow S_3$ represents an $M \rightarrow M^{ox}$ reaction. In the original model M was assumed to undergo redox reactions during $S_0 \rightarrow S_1$ and $S_3 \rightarrow S_0$. In the latter reaction M was assumed to become regenerated from M^{ox} during the course of a two electron oxidation of binuclearly complexed hydrogen peroxide [9,12]. This idea, which is attractive for energetical reasons can be reconciled with the spectral data of this study, if one assumes that the M/M^{0x}-redox couple undergoes the reaction twice during the cycle leading from H_2O to O_2.

Accordingly, $\Delta\varepsilon_{S_1 \rightarrow S_2}$ of Fig. 8 does not directly represent the difference spectrum for the oxidation of the binuclear manganese cluster, because this process is spectrally superimposed by M^{ox}-reduction. Likewise, $S_3 \rightarrow S_0$ implies reduction foboth, M^{ox} and the catalytic binuclear mangannese cluster. The conclusion that a functional group undergoes a redox transition in $S_1 \rightarrow S_2$ and $S_3 \rightarrow S_0$, but does not participate in $S_0 \rightarrow S_1$ and $S_2 \rightarrow S_3$ is in line with previous conclusions of Velthuys [7]. Recent studies of Lavergne [38,39] support this idea. In agreement with Dekker et al. [14] our data show that $\Delta\varepsilon_{S_i,S_{i+1}}$ is not vanishingly small around 300 nm for $i = 0$ and 2 [7,38,39]. Based on the above-mentioned considerations the following redox reaction sequence is proposed:



(2)

In this schematic description Mn symbolizes the binuclear manganese center of the catalytic site; with the index giving the number of oxidizing redox equivalents that are accumulated. Possible changes in the coordination sphere coupled with the redox transitions are indicated by circles and squares. It has to be emphasized that the scheme of Eqn. 2 is practically identical with that proposed by Goodin et al. on the basis of EXAFS-data [40], if one assumes that D_1H and D_2H are the same species (M).

Within the framework of the model summarized by Eqn. 2 four fundamental questions arise for the molecular mechanism of water oxidation that will be briefly discussed: (a) what is the nature of the catalytic site? (b) what is the redox state of manganese in $\boxed{Mn}$ and $\boxed{Mn}_{2(ox)}$? (c) what is the chemical nature of component M? (d) What is the protonation pattern in the different S_i-states?

Implications for the molecular mechanism of photosynthetic water oxidation

If one accepts that oxygen–oxygen bond formation is mechanistically the essential step in the redox sequence from water to dioxygen a binuclear cluster of appropriate geometrical structure is the minimum requirement. Accordingly, in the original model [32,33] a binuclear manganese cluster has been proposed to form the core of the catalytic site. Experimental data about the relation between manganese content and oxygen-evolving capacity have been used as argument for the catalytic site to be a tetranuclear manganese cluster [41,42]. However, based on the heterogeneity in the magnetic interaction with Z^{ox} [43] and in analogy to other metal enzymes catalyzing the reaction in the reverse direction, i.e., O_2-reduction to H_2O (for a review see Ref. 44) we conclude that the catalytic site for O–O-bond formation is a binuclear manganese complex. This idea is also supported by results obtained for in vitro model systems including manganese or other transition metal centers [45,46]. Information about the first coordination sphere of the binuclear manganese cluster is lacking. It is widely accepted that at least two water species (H_2O and/or OH^-) are directly associated with the manganeses of the catalytic site (for a review, see Ref. 1). Latest data confirm this conclusion [47].

The other two manganeses could provide the electronic coupling to Z^{ox} as discussed in Ref. 1. If one additionally assumes that electron transfer from Z^{ox} to these two manganeses is the rate-limiting step then this latter group undergoes a kinetically and spectroscopically 'silent' redox

transition that escapes our detection. Therefore other analytical tests could be required to clarify the possible role of these manganeses.

In respect to the redox state of manganese at the catalytic site, one should consider that charge delocalization of holes could be essential for stabilization of the redox intermediates of water oxidation [32,33]. Accordingly, in the oxidized state (S_2 and S_3) a redox equilibrium of the type

$$[Mn(n+1)(OH)^{-}(HO)^{-}Mn(m+1)] \rightleftharpoons \left[Mn(n) \begin{matrix} H \\ O \\ \\ O \\ H \end{matrix} Mn(m) \right] \quad (3)$$

has to be taken into account. This idea is in line with recent findings obtained in synthetic binuclear manganese complexes [48]. The latest EPR-experiment performed in samples suspended in ^{17}O-enriched water do not exclude the possibility of a μ-peroxobridged binuclear manganese center in S_2 [47]. The existence of redox equilibria between metal centers and water substrate ligands has two important implications: (i) Conclusions drawn from low-temperature EPR-spectra of S_2 do not necessarily reflect the in vivo situation unless the electronic charge density distribution is shown to remain temperature independent. Recently, the possibility of temperature-dependent electronic metal-ligand transitions have been reported for synthetic manganese-quinol-complexes [49]. (ii) Information about the formation of peroxide (superoxide) configurations at the catalytic site in S_2 and S_3 can be obtained by mass spectroscopic detection of the evolved dioxygen only if effects due to Eqn. 3 and water ligand exchange in redox state $Mn(n+1)(OH)^{-}(HO)^{-}Mn(m+1)$ are negligible. The values of n and m in Eqn. 3 are very likely II and III, respectively [37,40,47,48]. Referring to the nature of M it has to be stressed that the difference spectrum that is assumed to reflect M-oxidation does not permit an unambiguous substantiation. It does neither exclude the participation of a special quinone system as discussed recently (see Ref. 50) nor the involvement of a further transition metal redox center, i.e., the other two manganeses (vide supra). Further experiments are required to clarify this point.

For a summarizing scheme informations are required about the deprotonation pattern. It has been thoroughly discussed [33] that the extrinsically measured H^+-release pattern does not necessarily reflect the actual deprotonation step at the catalytic site. For the sake of simplicity possible effects due to the apoenzyme will be neglected here and the widely accepted stoichiometry for extrinsic H^+-release [51] is used. Accordingly, either M^{ox}-formation itself or an electrostatic effect of M^+ on a deprotonizable group in the surrounding gives rise to H^+-release. Keeping these alternatives in mind, we will symbolize this deprotonation by the reaction: $MH \rightarrow M^{ox} + + H^+$. If furthermore the binuclear manganese cluster of the catalytic site attains a mixed valence state of

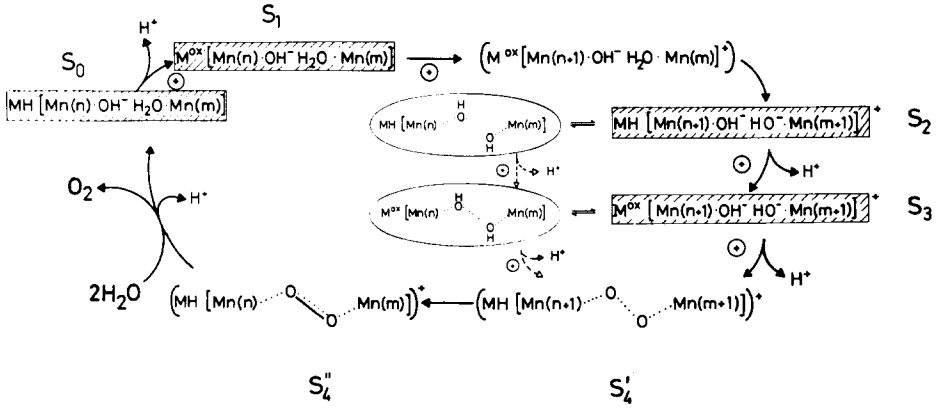


Fig. 11. Model of photosynthetic water oxidation. For details see text. The charges indicated at the catalytic site are related to S_0 as reference.

[Mn(II) Mn(III)] in its reduced form (S_0/S_1) (see Ref. 40) it appears likely to assume that water binding to Mn(III) leads to deprotonation [52]. Summarizing the considerations, the mechanism of photosynthetic water oxidation can be described by the scheme depicted in Fig. 11.

Acknowledgements

The authors would like to thank M. Völker for the PS-II-membrane fragments, B. Hanssum for reading the manuscript and A. Schulze for drawing the figures. The financial support by Deutsche Forschungsgemeinschaft (SFB 312) is gratefully acknowledged.

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