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Minireview

Reaction pattern and mechanism of light induced oxidative water splitting in photosynthesis

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Abstract

This mini review is an attempt to briefly summarize our current knowledge on light driven oxidative water splitting in photosynthesis. The reaction leading to molecular oxygen and four protons via photosynthesis comprises thermodynamic and kinetic constraints that require a balanced fine tuning of the reaction coordinates. The mode of coupling between electron (ET) and proton transfer (PT) reactions is shown to be of key mechanistic relevance for the redox turnover of Y_Z and the reactions within the WOC. The WOC is characterized by peculiar energetics of its oxidation steps in the WOC. In all oxygen evolving photosynthetic organisms the redox state S_1 is thermodynamically most stable and therefore this general feature is assumed to be of physiological relevance. Available information on the Gibbs energy differences between the individual redox states S_{i+1} and S_i and on the activation energies of their oxidative transitions are used to construct a general reaction coordinate of oxidative water splitting in photosystem II (PS II). Finally, an attempt is presented to cast our current state of knowledge into a mechanism of oxidative water splitting with special emphasis on the formation of the essential O–O bond and the active role of the protein environment in tuning the local proton activity that depends on time and redox state S_i. The O–O linkage is assumed to take place within a multistate equilibrium at the redox level of S_3, comprising both redox isomerism and proton tautomerism. It is proposed that one state, $S_3(P)$, attains an electronic configuration and nuclear geometry that corresponds with a hydrogen bonded peroxide which acts as the entatic state for the generation of complexed molecular oxygen through $S_3(P)$ oxidation by Y_Z^{ox}.

Keywords: Photosystem II; P680; Redox active tyrosine; Proton coupled electron transfer; Water splitting

1. Introduction

The "invention" of a functional unit that enabled light-induced oxidative water splitting into molecular oxygen and four protons by photosynthesis was the cornerstone in the evolutionary development of solar energy exploitation as the unique Gibbs energy source of living matter. This "big bang" in bioenergetics which occurred 2.5–3 billions years ago at the level of ancient cyanobacteria [1–4] had two consequences of paramount importance: (i) the huge water pool on earth surface became available as hydrogen source for the biosphere, and (ii) oxygen released as the "waste" product enriched the atmosphere to the present day aerobic level [5,6] thus opening the road for much more efficient (more than a factor of ten) exploitation of the Gibbs energy content of food through the pathway of aerobic respiration (for thermodynamic considerations, see [7]). Furthermore the availability of huge amounts of molecular oxygen led to the generation of the stratospheric ozone layer as the protective "umbrella" against deleterious UV-B effects.

The essential steps of oxidative water splitting take place within the multimeric protein complex of photosystem II (PS II) that acts as water:plastoquinone oxidoreductase. This complex consist of more than 20 subunits [8] and is characterized by a composition of both, cofactors and polypeptides, that is surprisingly invariant to evolutionary development [9]. The PS II complex is therefore Nature's unique masterpiece for biological solar energy exploitation by photosynthetic water splitting.

The overall process of PQ reduction with water as hydrogen donor in PS II comprises three different types of reaction sequences: (i) light induced stable charge separation leading to formation of the radical ion pair $P680^{+\bullet}Q_A^{-\bullet}$ (for a review, see [10]), (ii) oxidative water splitting into molecular oxygen and

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four protons energetically driven by $P680^{+\bullet}$ (the topic of this review) and (iii) reduction of plastoquinone to quinol under proton uptake with $Q_A^{-\bullet}$ acting as reductant (for review, see [11]).

Within this series of events only the nature of P680 and the oxidative water splitting are unique features of PS II. The indispensable prerequisites for this process are the generation of sufficiently oxidizing redox equivalents and the existence of a catalytic device which is able to perform the controlled cooperative reaction of four electron holes with two substrate molecules under formation of molecular oxygen and four protons.

The first goal was achieved by tailoring a chlorophyll a (Chl a) containing complex into a specific protein matrix. This specific pigment complex is denoted P680. Electronic singlet state excitation of P680 leads to electron ejection under formation of the cation radical $P680^{+\bullet}$ which is one of the strongest oxidants in biology. The reduction potential of P680 was estimated to be about +1.25 V [12]. Details of light induced $P680^{+\bullet}$ formation and the tuning of its oxidation power has been outlined in a recent review and will not be discussed here (see [10]).

This contribution is an attempt to briefly summarize our knowledge on the energetic and kinetic pattern of the reaction sequence:

$$2H_2O + 4\oplus(P680^{+\bullet}) \rightarrow 4H^{+\bullet} + O_2 \quad (1)$$

with the challenging goal of understanding the underlying mechanism.

2. Kok cycle

The pioneering work of Joliot and Kok and their coworkers provided a unique fingerprint for unravelling the reaction summarized in Eq. (1). Illumination of dark adapted algae and isolated chloroplasts by a train of single turnover flashes generates a pattern of the oxygen yield per flash that is characterized by damped period four oscillation with maximum O_2 release after the 3rd, 7th, 11th, 15th etc. flash [13,14]. Another most important result of these studies was the observation that the characteristic oscillation is invariant to the number of fully competent PS II complexes. These findings imply that oxidative water cleavage in PS II is a four-step sequence of redox reactions which comprises the intermediary storage of oxidizing redox equivalents (electron holes). After accumulation of four electron holes and formation of molecular oxygen, the system returns to its initial state and the next cycle starts. This reaction scheme was first proposed by Kok and coworkers [14] and is therefore denoted "Kok cycle". The storage states are symbolized by S_i where index i represents the number of stored holes relative to the lowest redox state S_0 of the WOC under turnover conditions with water as substrate. The pronounced maximum of the oxygen yield after the 3rd flash shows that redox state S_1 is most stable and populated after sufficient dark adaptation.

Table 1
Half life times [s] of S_0 oxidation by Y_D^{OX} and S_2/S_3 reduction by Y_D

Reaction	Cyanobacteria		Higher plants
	Acaryochloris marina	*Thermosynechococcus elongatus*	*Spinacea oleracea*
$Y_D^{ox}S_0 \rightarrow Y_D S_1$	125	2300	580
$Y_D S_2 \rightarrow Y_D^{ox} S_1$	5.5	0.8	2.0
$Y_D S_3 \rightarrow Y_D^{ox} S_2$	5.2	1.0	1.7

Subsequent studies revealed that the original Kok scheme has to be extended: (a) the stepwise oxidation of the WOC by $P680^{+\bullet}$ is mediated by a redox active component (symbolized by Y_Z) [15] and (b) redox states below S_0 can be populated by chemical means (states down to S_{-3} are well characterized, see [16] and references therein, and even the existence of S_{-4} and S_{-5} cannot be excluded, as discussed in [17]) and are even formed *in vivo* [18].

The component Y_Z was identified as Y161 of polypeptide D1 [19,20]. The PS II complex contains two redox active tyrosines: Y_Z (in polypeptide D1) and Y_D (in polypeptide D2) which are spatially arranged symmetrically to P680 but characterized by quite different properties (for a recent review, see [21]). The driving force of the conventional Kok cycle by $P680^{+\bullet}$ is exclusively mediated by Y_Z. The strikingly lower redox potential of Y_D/Y_D^{OX} [22] and the much larger distance to the WOC give rise to an entirely different reaction behaviour, i.e. S_2 and S_3 reduction by Y_D and the very slow oxidation of S_0 by Y_D^{OX} [23,24]. Table 1 compiles half life times of these reactions in different organisms. An extended Kok scheme is shown in Fig. 1.

3. Oxidation of tyrosine Y_Z (and Y_D) by $P680^{+\bullet}$

The energetics of tyrosine oxidation strongly depends on the protonation state of its phenolic OH group which exhibits *in vitro* pK-values of about 12 and about −2 for the reduced and oxidized form, respectively [25] Therefore the driving force of both, Y_Z oxidation by the cation radical $P680^{+\bullet}$ and the S_i-state transitions by Y_Z^{ox}, are intimately related to proton transfer reactions within a protein environment. The mode of coupling between electron transfer (ET) and proton transfer (PT) is a fundamental problem in reaction kinetics and the topic of several theoretical studies [26–30]. In enzyme systems protonatable groups of the protein environment can severely affect the reaction pathway via formation of hydrogen bonds as outlined for oxidation of phenolic groups in model systems [31]. Different types of proton coupled electron transfer (PCET) are distinguished with great variations of coupling strength and distances between the redox groups (for a review see [32]).

The kinetics of $P680^{+\bullet}$ reduction by Y_Z are surprisingly complex. In a reasonable approximation, the overall time course of this process is satisfactorily described by three exponential kinetics with "fast" ns, "slow" ns and μs components. This general feature was observed in thylakoids [33], PS II membrane fragments [34–36] and PS II core complexes from both, spinach and thermophilic cyanobacteria [37,38]. Indirect lines of evidence suggest that it also pertains to intact leaves (*Arabidopsis thaliana*) [39]. These kinetics markedly depend on

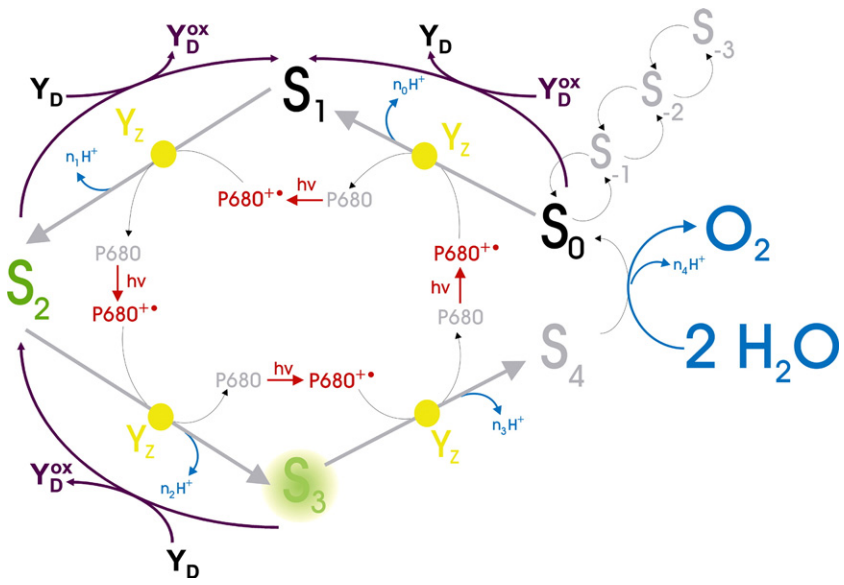

Fig. 1. Extended Kok-cycle of oxidative water cleavage. The photo oxidation leading to $P680^{+\bullet}$ (marked in red) is symbolized by arrows with hν, Y_Z by a yellow dot and the S_i-states by capital letters in different colors: the dark stable redox state S_1 is in black, the metastable redox state S_2 and S_3 in green without and with, respectively, a dark green background, the transient "elusive" state S_4 and the super reduced states are marked in grey. The reactions with the redox couple Y_D^{OX}/Y_D are symbolized by violet arrows. For the sake of simplicity the slow dark relaxation reactions of S_2 and S_3 are omitted (for a review, see [78,153]).

the redox state of the WOC in all samples (see below) and change drastically in systems lacking the WOC [34,35,40]. Basically, two alternative explanations can be considered for the origin of the multiphasic kinetics in systems with an intact WOC as outlined in [37]: (i) "static" sample heterogeneity (e.g. an ensemble of PS II complexes with different distances between Y_Z and P680 or energetic parameters like Gibbs and/or reorganization energy) or (ii) transition through a sequence of redox equilibria of the type $[P680^{+\bullet}\,Y_Z \Leftrightarrow P680\ Y_Z^{OX}]_j$ due to relaxation processes analogous to those discussed for the radical pairs of charge separation (for details see [10]), where index j symbolizes one state of the relaxation sequence. A critical survey of the experimental data favours the idea of a sequence of relaxation states [37]. The initial step is characterized by rate constants of $(20–50\ ns)^{-1}$ [33–39], activation energies of 10–20 kJ/mol [35,37,31] and a vanishingly small kinetic H/D isotope effect [42]. Eckert and Renger [35] proposed that this fast reaction comprises the transfer of an electron from Y_Z to $P680^{+\bullet}$ coupled with a proton shift within a hydrogen bond formed between the OH group of Y_Z and a nearby base X. Based on site directed mutagenesis studies (see [43] and references therein) and X-ray diffraction crystallography (XRDC) data [44,45] base X is identified as His 190 of polypeptide D1. Analysis of the data obtained for dark adapted samples with WOC in redox state S_1 revealed that the rate constant of $P680^{+\bullet}$ reduction by Y_Z during equilibration within the initial state I can be consistently described by the Marcus theory of nonadiabatic electron transfer [46] with a reorganization energy of about 0.5 eV [47,48]. Therefore the redox process in the initial state I of non-relaxed microenvironment is inferred to be kinetically limited by the electron transfer step. The relaxation sequence model implies that the rate constant k_1 for $P680^+$ reduction by Y_Z is given by the relation $k_1 = k_{obs} \times K_{eq}(I)/(K_{eq}(I)+1)$ where k_{obs} is the experimentally measured rate constant and $K_{eq}(I)$ the equilibrium constant in state I [37].

The extent of the "fast" ns kinetics normalized to the total $P680^{+\bullet}$ reduction by Y_Z is large in redox states S_0 and S_1 of the WOC and significantly smaller in S_2 and S_3 [34–36]. This dependence reflects the energetic effect of the redox states S_i of the WOC on the equilibrium constant in the initial state $[P680^{+\bullet}\ Y_Z \Leftrightarrow P680\ Y_Z^{OX}]_I$ and could originate either from an electrostatic effect due to the presumed positive charge in S_2 and S_3 [49,50] or from conformational changes [51]. The kinetics of the "slow" ns components with rate constants of $(300–600\ ns)^{-1}$ are invariant to replacement of exchangeable protons by deuterons [42] and the activation energy is somewhat larger (a factor of 1.5–2) than that of the "fast" ns-kinetics [37,41]. The normalized amplitudes exhibit a dependence on the redox state S_i of the WOC which is opposite to that of the "fast" ns component, i.e. higher values in S_2 and S_3 and lower values in S_0 and S_1 [34–36] These kinetics are assumed to reflect the response dynamics of the nearest protein environment to the redox process which shifts the equilibrium constant to higher values. The lack of any kinetic isotope exchange effect suggests that this relaxation step does not comprise "large scale" hydrogen bond rearrangements [37]. In marked contrast, the component with 30–35 μs kinetics exhibits a pronounced H/D exchange effect [36,52,53] and therefore is assigned to the rearrangement of a hydrogen bond network within the environment of Y_Z. The energetics of the different relaxation steps are sensitive to environmental changes as reflected by a dependence on the sample type and will be discussed in a forthcoming paper (Kühn and Renger, unpublished results). The interpretation of the multiphasic $P680^{+\bullet}$ reduction kinetics by Y_Z via a sequence of relaxation steps implies that the protein matrix plays an active role in

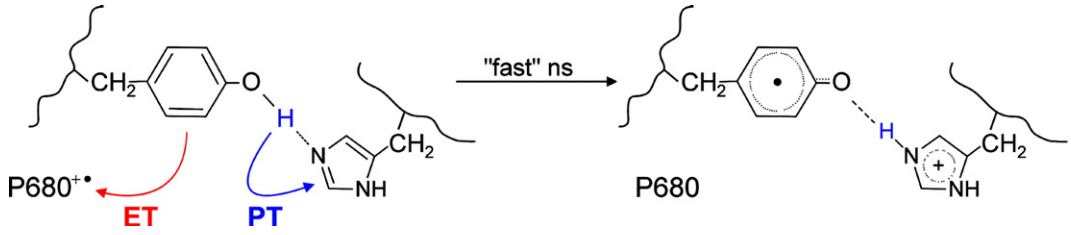

Fig. 2. Scheme of proton coupled electron/proton transfer of $P680^{+\bullet}$ reduction by Y_Z with His as hydrogen bonding partner. Electron transfer (ET) and proton transfer (PT) are symbolized by red and blue, respectively, characters (for details, see text).

$P680^{+\bullet}$ reduction by Y_Z. For energetic reasons the redox reaction $P680^{+\bullet}$ $Y_Z \rightarrow P680$ Y_Z^{OX} requires a removal of the phenolic proton from the tyrosine [54]. Therefore, questions arise about the mode of coupling between electron and proton transfer. As a consequence of the proposed hydrogen bond between the OH group of Y_Z and His 190 the reaction is assumed to occur via a concerted mechanism where the orbitals involved in ET and PT belong to different atoms. This process is illustrated by the scheme of Fig. 2.

The initial PT from Y_Z to His 190 gives rise to transient formation of an imidazolium cation, followed by a sequence of PT steps where the proton is either eventually released into the lumen as suggested by Babcock and coworkers (see [55] and references therein) or trapped inside the protein matrix [56]. The nature of the hydrogen bond between Y_Z and His 190 is critical for the kinetics of Y_Z oxidation by $P680^{+\bullet}$. Therefore the possibility of a low barrier hydrogen bond (LBHB) configuration has been discussed [57,58]. At present a confirmation of this idea is lacking.

A protonation of His 190 is expected to disrupt the essential hydrogen bond and therefore the normalized extent of the "fast" ns kinetics should decrease upon lowering the pH of sample suspensions. This was really found to be the case and the results obtained for PS II core complexes from *Thermosynechococcus* (*T.*) *elongatus* can be described by a single protonatable group with a pK of 4.6 [59]. A value <5 is markedly lower than the pK of His in solution which is about 6.0 (see textbooks of Biochemistry). Significant shifts of pK values of amino acid residues in proteins are not unusual, in particular when hydrogen bonds are involved. Furthermore, DFT calculations revealed that the pK of His also depends on the conformation (angle of the imidazole ring plane) with possible variations by about two pH units [60].

As an alternative, the pH effect could be also explained by Ca^{2+} release as discussed in a former report [61]. Evidence for a Ca^{2+} (Sr^{2+}) specific effect on the kinetics of $P680^{+\bullet}$ reduction by Y_Z have been reported in the literature [51,61,62] but the origin of this effect is a matter of debate.

In PS II complexes without a functionally competent WOC the reaction pattern of $P680^{+\bullet}$ reduction by Y_Z drastically changes: the ns kinetics disappears in the physiological pH range and the reaction is dominated by μs kinetics with a

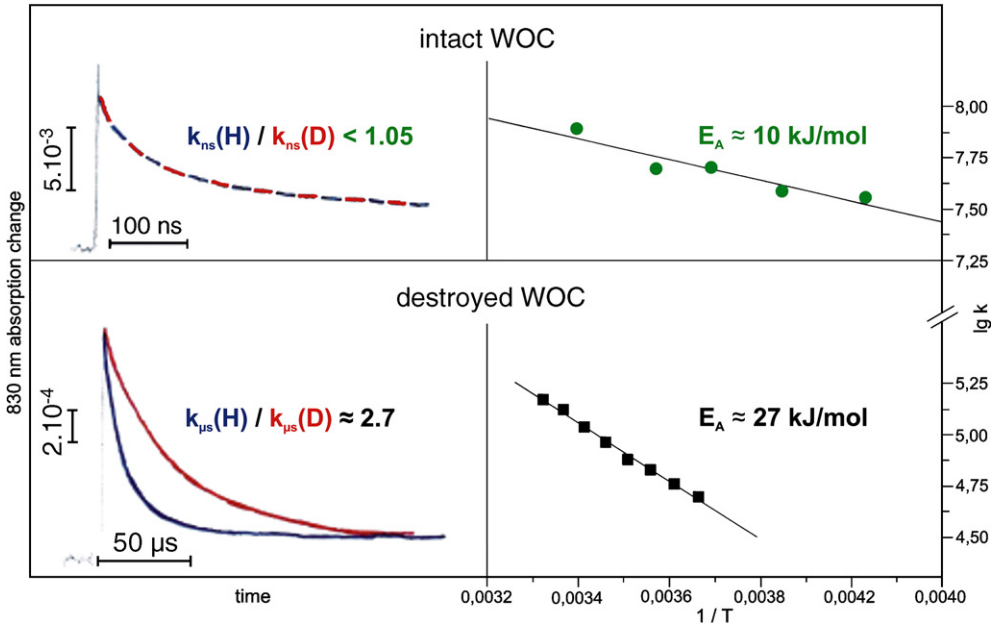

Fig. 3. Flash induced 830 nm absorption changes (left side) and rate constants of the "fast" ns kinetics as a function of reciprocal temperature (right side) in systems with (top panels) and without (bottom panels) a fully competent WOC (for details, see text).

significantly larger (30–40 kJ/mol) activation energy [47,63,64] and a pronounced H/D exchange effect of about 3 [64–66] as shown by a compilation of typical data in Fig. 3. Furthermore, the oxidation of Y_Z in these samples is coupled to a stoichiometric proton release into the lumen [67] and a drastic increase of the reorganization energy from about 0.5 eV (see above) to about 1.6 eV [47,48]. A similar value of 1.4 eV was reported by Sjödin et al. [31]. These findings indicate that the reaction coordinate is significantly altered in PS II complexes without a functional WOC. Changes take place in the environment of Y_Z including the penetration of water molecules into the microenvironment [47,48,58] and probably modifications of hydrogen bonding (for a review, see [68]).

$P680^{+\bullet}$ also oxidizes the tyrosine Y_D in the polypeptide D2 with a striking pH dependence of its kinetics [69,70]. However, the reaction of Y_D/Y_D^{OX} with the WOC is entirely different as illustrated in Fig. 1 (for further details, see Section 6, Fig. 4 and Table 1).

4. Reaction pattern and properties of the water oxidizing complex (WOC)

The Kok cycle *per se* provides only a formal description of the four step reaction sequence energetically driven by the strongly oxidizing cation radical $P680^{+\bullet}$ and with Y_Z acting as intermediary redox carrier. Therefore a deeper mechanistic understanding of oxidative water cleavage requires information on: (i) the structure of the WOC, (ii) the electronic configuration and nuclear geometry of the catalytic site in each redox state S_i and (iii) the reaction coordinates of the individual redox steps.

4.1. Structure of the WOC

Among the numerous metalloenzymes the WOC is unique because its assembly occurs via a special light driven reaction sequence denoted photoactivation [71–74]. "Superreduced" states (see Section 2 and Fig. 1) are inferred to be intermediates of this process [75,76] The feature of photoactivation reveals that the WOC is thermodynamically a metastable system that requires protection by the protein matrix against reductive degradation.

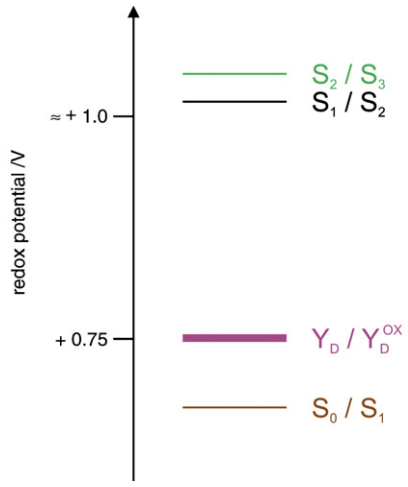

Fig. 4. Redox potential diagram of S_o/S_1, S_1/S_2, S_2/S_3 and Y_D/Y_D^{ox} (for details, see text). The position of the +1.0 eV level has an uncertainty of at least 0.1 eV (see text).

Different lines of biochemical and spectroscopic evidence indicate that the catalytic site of the WOC is a Mn_4O_XCa cluster, where x denotes the number of μ-oxo-bridges (for recent reviews, see [77,78]). The crucial role of a well defined array and binding of this cluster into a suitable matrix is nicely illustrated by model systems where the synthetic manganese complex $[(H_2O)_2(terpy)_2Mn_2(\mu\text{-}O)_2]^{3+}$ must be properly adsorbed on mica or kaolin in order to catalyze water oxidation to molecular oxygen with Ce^{4+} as oxidant. The same metal complex in solution is entirely inactive as catalyst and prone to dissipative oxidation to MnO_4^- [79]. The structure of the WOC is defined by (a) the spatial arrangement of the four manganese centers, the bridging oxygens and the Ca^{2+} ion, (b) the coordination sphere of the Mn_4O_XCa cluster and (c) the surrounding protein matrix.

At present a picture of the WOC at atomic resolution is lacking. The XRDC structures with the highest resolution of 3.5 Å [44], 3.2 Å [80] and 3.0 Å [45] comprise proposals for the geometry of the manganese cluster including amino acid ligands. However, for two reasons this structural information does not offer a sound basis for mechanistic considerations on oxidative water cleavage: (a) the limited resolution of 3.0 Å does neither resolve the oxygen atoms of the μ-oxo bridges and of the bound substrate molecules nor does it provide information on possible hydrogen bond networks and (b) the XRDC analyses are generally faced with serious problems that originate from the use of the required high doses of X-ray radiation. This leads to "radiation damage" effects owing to the generation of photoelectrons which interact with several amino acid side chains and in particular with reducible transition metal centres [81]. The latter effect is of special relevance for the Mn_4O_xCa cluster of the WOC. EXAFS studies performed at much weaker X-ray radiation (factor of about 1000) and at 10 K unambiguously showed that the manganese is reduced to Mn(II) at the doses used for XRDC analyses and that all Mn–Mn distances and Mn–O–Mn bridges are lost [82]. In order to obtain reliable structural models, future X-ray crystallographic studies must aim at achieving experimental conditions, which permit the use of drastically reduced radiation doses and simultaneously to improve the spatial resolution.

Precise data on Mn–Mn and Mn–O distances and on the number of these structural elements within the Mn_4O_XCa cluster were obtained by using X-ray spectroscopy. The seminal work of Mel Klein, Ken Sauer, Vittal Yachandra and coworkers revealed that the Mn_4O_XCa cluster is characterized by two or three Mn–Mn distances of about 2.7 Å and one Mn–Mn distance of about 3.3 Å (for a review, see [83]). Extended X-ray absorption fine structure (EXAFS) studies also revealed that significant structural differences exist between different S_i-states [84,85]. Several models for the geometrical array of Mn, O and Ca^{2+} are consistent with this distance information (for a

review, see [77]). A significant reduction of this number to three possible models was achieved by measurements of polarized EXAFS on single crystals of PS II core complexes from *T. elongatus* [86]. It is important to note that these three models differs from the structures gathered from XRDC studies of Ferreira et al., and Loll et al. [44,45]. A combination of these results with XRDC data leads to suggestions for a likely coordination of the Mn_4O_xCa cluster by the protein matrix [86] but the proposed ligand array is still questionable because the protein environment is expected to be also modified as a consequence of radiation damage effects.

Besides the connection via μ-oxo bridges, the manganese and Ca^{2+} ions are coordinated by other ligands, in particular by amino acid residues (containing carboxylic and imidazol groups) and most importantly by the interaction sites with the substrate molecules. Site directed mutagenesis studies [87] and XRDC structure analyses [44,45] identified Asp 170, His 332, Glu 333, Asp 342 and the carboxyl terminus of Ala 344 of polypeptide D1 as ligands of the Mn_4O_XCa cluster. Furthermore, Glu 354 of CP43 was also shown to be an essential constituent of the WOC [88] and based on XRDC data proposed to be a manganese ligand [44,45]. Differences exist with respect to His 337 and Glu 189 of polypeptide D1. FTIR spectroscopy offers a most powerful tool to probe ligand–metal interaction (for a review, see [89]). The following ligand assignments were reported: (i) Asp 170 and His 332 bind to manganese which is not oxidized in the conventional Kok cycle up to S_3 [90,91] but at least in the case of His 332 it is sensitive to structural changes of the Mn_4O_XCa cluster coupled with the S_i-state transitions of the WOC [91], (ii) the carboxyl group of the C-terminus of polypeptide D1 (Ala 344) coordinates to a Mn that is the site of oxidation in the $S_1 \rightarrow S_2$ transition and of rereduction in the $S_3 \rightarrow S_0$ transition [92,93] and (iii) Glu 189 is most likely not a direct ligand to the Mn_4O_xCa cluster but coordination to a manganese which does not change its redox state during the Kok cycle cannot be entirely ruled out [93,94]. However, it must be emphasized that straightforward interpretation of the FTIR data is difficult due to uncertainties on the exact relationship between protein polarisability and changes of the oxidation state of Mn_4O_xCa.

Of central relevance for mechanistic considerations is a detailed knowledge on the binding sites for the substrate molecules and the mode of their hydrogen bonding. Direct structural information is lacking (see above). Indirect lines of evidence for binding of water molecules to Mn_4O_XCa were gathered from electron-nuclear double resonance (ENDOR) [95] and electron-spin echo envelope modulation (ESEEM) studies [96,97]. FTIR measurements revealed the existence of water molecule(s) with asymmetric hydrogen bonding of the two protons in the S_1 state. This pattern changes to even higher asymmetry when the WOC becomes oxidized from S_1 to S_2 [98]. Likewise the redox transitions $S_0 \rightarrow S_1$, $S_2 \rightarrow S_3$ and $S_3 \rightarrow \rightarrow S_0$ were shown to give rise either to strong hydrogen bond interaction or to proton release [99]. However, an unambiguous assignment of specific substrate molecules bound to the Mn_4O_XCa cluster could not be achieved so far. It is expected that future FTIR studies with suitable isotope labelling will provide this information. The exchange kinetics of substrate water molecules with the catalytic site in the different redox states S_i were monitored via $H_2^{18}O/H_2^{16}O$ exchange experiments (for detailed discussion see [78,100]). It must be emphasized that several problems of high mechanistic relevance are still unresolved, like the coordination of the substrate to specific metal centers and in particular the mode of hydrogen bonding.

In addition to amino acid residues and substrate molecules inorganic ligands like Cl^- and HCO_3^- are discussed as possible members of the first coordination sphere (for reviews, see [101,102]). HCO_3^-, which plays an important role in PS II (for a review see [103]), was modelled into the XRDC structure proposed by Ferreira et al. [44], but more refined data are not in favour with this suggestion [45]. Likewise, FTIR studies do not support the idea of direct HCO_3^- ligation [94]. With respect to Cl^-, a possible signature has been reported for binding to Mn based on EXAFS studies on oriented samples with the WOC in redox state S_3 [77]. On the other hand, FTIR data led to the conclusion that Cl^- is not a direct ligand to Mn [104]. This conclusion is supported by X-ray spectroscopy on bromine reconstituted PS II membrane fragments [105]. Therefore, at present straightforward experimental evidence for direct binding to the Mn_4O_XCa cluster exists for neither of these two species.

4.2. Electronic configuration and nuclear geometry of S-states

For the sake of clarity the S_i-states will be described by a triple symbol $S_i = M_jL_kW_l$ with $i=j+k+l$, where M_j, L_k and W_l reflect the formal redox states of the manganese (M), the non-substrate ligand (L) and the substrate (W), respectively. In some cases it is of advantage to summarize the contributions of either the oxidation states of M and L, i.e. $S_i = (ML)_mW_l$ with $m=j+k$, or L and W, i.e. $S_i = M_j(LW)_n$ with $n=k+l$. The numbers j, k, l, m and n are used as integers to symbolize metal-centered (j), non-substrate ligand-centered (k) and substrate-centered (l) redox steps in the WOC. It must be emphasized that these formal numbers (j…n) are related to the actual electronic structure of each individual Si state but do not reflect the actual valence state of manganese and its first coordination sphere.

The electronic configuration of the Mn_4O_XCa cluster can be best probed by X-ray absorption near edge (XANES), EPR and ^{55}Mn ENDOR spectroscopy. The results of studies using these techniques lead to the following conclusions: (a) the overall manganese valence state of Mn_4O_XCa in the dark adapted redox form S_1 of the WOC is most likely $Mn(III)_2$ $Mn(IV)_2$ [106,107], (b) the redox transitions $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_2$ are metal centered reactions (for a review, see [77]), (c) the nature of the reaction Y_Z^{OX} $S_2 \rightarrow Y_zS_3$ is not yet resolved, but based on different lines of evidence (for reviews, see [108–110]) this seems to be likely a ligand-centered oxidation (for further discussion see [78]) and (d) the nature of the kinetically elusive transient state S_4 is a matter of controversial discussions and speculations (see Section 6).

5. Reaction coordinate of oxidative water cleavage

The reaction coordinate is determined by the energetics of the S_i-states and the activation energies for the advancement of the WOC from the redox level of substrate water to molecular oxygen and four protons.

5.1. Energetics of the S_i-states

Compared with the four step sequence of oxidative water cleavage into molecular oxygen and four protons in aqueous solution [111] the energetics of the Kok cycle are drastically different (for a review, see [112]). Absolute values of the S_i-state energy levels of the normal Kok cycle ($i=0, \ldots 4$) are not known. Likewise, a direct experimental determination of the standard Gibbs energy differences between redox states S_i and S_{i+1}, $\Delta G°(S_{i+1}/S_i)$, cannot be achieved. However, estimations of these values are obtained on the basis of the relation:

$$\Delta G°(S_{i+1}/S_i) = \Delta G(P680^{+\bullet}/P680) - \Delta G°(P680Y_Z^{OX}S_i/P680^{+\bullet}Y_ZS_i) - \Delta G°(Y_ZS_{i+1}/Y_Z^{OX}S_i)$$

where $\Delta G°(P680^{+\bullet}/P680)$, $\Delta G°(P680Y_Z^{ox}S_i/P680^{+\bullet}Y_ZS_i)$ and $\Delta G°(Y_ZS_{i+1}/Y_Z^{ox}S_i)$ are the Gibbs energy differences for the formation of the cation radical $P680^{+\bullet}$, oxidation of Y_Z by $P680^{+\bullet}$ and S_i-state transitions, respectively.

Using data from the literature, the values of $\Delta G°(S_{i+1}/S_i)$ are calculated to be about 0.85 eV, 1.10 eV, 1.15 eV and 1.0 eV for $i=0$, 1, 2 and 3 as outlined in [78]. It is important to note that a value of 0.85 eV for the Gibbs energy gap between S_1 and S_2 is not in line with the reported redox potential of about +0.75 V for Y_D/Y_D^{OX} [74,113] because Y_D^{ox} is known to oxidize S_0 to S_1 (see Fig. 1). This finding indicates that further studies are required to unravel the details of the energetics of the $S_i \rightarrow S_{i+1}$ transitions.

A consequence of the slow S_0 oxidation by Y_D^{ox} (see Table 1) is the virtual absence of S_0 populations in dark adapted samples. Interestingly, this feature is found in all oxygen evolving organisms [114–116] including also the Chl *d* containing cyanobacterium *Acaryochloris marina* [117] (see Table 1) and therefore it seems to be most likely of relevance in suppressing deleterious reactions that are not yet clarified (for further considerations, see [118]).

Furthermore, an extra Gibbs energy gain of about 0.25 eV emerges for the $Y_Z^{ox}S_0 \rightarrow Y_ZS_1 + n_0H^+$ transition compared to the other redox steps. The physiological role of this peculiarity remains to be unravelled. It has been speculated that this surplus of Gibbs energy could be transiently stored [119,120] but so far experimental evidence is lacking for this idea.

The last step of oxidative water cleavage is the product/substrate exchange. In earlier model considerations this reaction was assumed to be significantly exergonic with values of 100–200 meV [121]. New data on the back pressure effect of molecular oxygen, however, suggested that the reaction $Y_Z^{ox}S_3 \Leftrightarrow [Y_ZS_2(H_XO_2)] \Leftrightarrow [Y_ZS_0(O_2)] \Leftrightarrow Y_ZS_0 + O_2 + n_3H^+$, which comprises the product/substrate exchange step, is only slightly exergonic [122], where the nature of S_3 with respect to possible ligand/substrate oxidation (see section 6) is not specified and x symbolizes the unknown protonation state of complexed peroxide.

Although precise values are missing for $\Delta G°(S_{i+1}/S_i)$ due to experimental uncertainties (vide supra) the energetic considerations reveal that water cleavage is thermodynamically possible down to local pH values of 5.0.

5.2. Kinetics and activation energies of S_i-state transitions

The results of time resolved flash induced transients of EPR spectroscopy [123,124] and optical UV absorption changes [125–127] showed that the kinetics of Y_Z^{OX} reduction and WOC oxidation are virtually identical, thus indicating that Y_Z^{OX} is the direct oxidant of the Mn_4O_XCa cluster. XRDC studies confirm this conclusion [44,45]. Closer inspection of the data reveals several striking features: (i) the kinetics of the reactions $Y_Z^{ox}S_i \rightarrow Y_ZS_{i+1} + n_iH^+$ are similar for $i=0$, 1 and 2 (variation by factors of 2 up to 4) while the reaction $Y_Z^{ox}S_3 \rightarrow Y_ZS_0 + O_2 + n_3H^+$ is markedly slower by factors of 5 up to 15 compared to the former reactions, (ii) the signal that reflects the redox reaction between Y_Z^{ox} and S_3 exhibits a sigmoidal time course, and (iii) the $Y_Z^{ox}S_3$ decay kinetics are particularly sensitive to different types of sample modifications, e.g. replacement of Asp 61 of polypeptide D1 in *Synechocystis sp.* PCC 6803 mutants by either Ala or Asn gives rise to a kinetic retardation by factors of about 8 and 10, respectively [128].

Replacement of Ca^{2+} by Sr^{2+} retards the kinetics in samples from *T. elongatus* and higher plants [54,129]. It is concluded that Sr^{2+} gives rise to modifications of both, structure (most likely including hydrogen bond networks) and fine tuning of the redox properties [94,129].

The lag phase phenomenon was observed in several studies [125–127,130] and ascribed to a proton shift preceding the redox step [127]. However, it is not yet clear as to what extent the observed lag phase is a real property of the reaction or mainly the result of overlapping signals with opposite sign due to the intrinsic probability of misses in the Kok cycle [131]. A numerical analysis [132] led to the conclusion that a recently reported lag phase of about 250 μs [133] is likely due to artefacts since these experiment were performed on partially dried samples where the S_2 and S_3 oxidation by Y_Z^{ox} are progressively blocked [99]. This conclusion is supported by a comparative study on the kinetics of Y_Z^{ox} reduction and oxygen release where a lag phase – if it exists at all – was inferred to be 50 μs at most under normal in vivo conditions [134].

The activation energies (E_A) exhibit a close similarity of the values gathered from different sample material from thermophilic cyanobacteria and higher plants [131,135,136]. This striking feature indicates that the reaction coordinate of oxidative water cleavage remained virtually invariant to evolutionary development of PS II. Based on the $\Delta G°(S_{i+1}/S_i)$ and E_A values a general scheme is constructed for the reaction

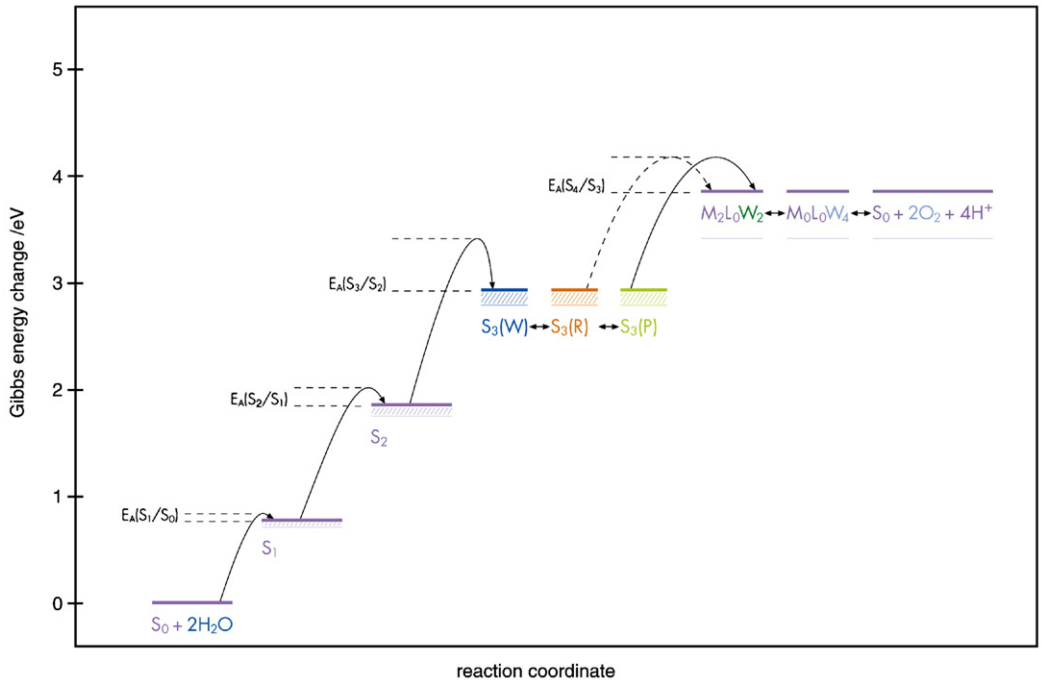

Fig. 5. Generalized reaction coordinates of the four step oxidative water cleavage in the photosynthetic apparatus. The three states of the redox isomerism in S_3 are symbolized by different colors and the energetic uncertainties by marked areas (for details, see Fig. 6 and text).

coordinate of oxidative water splitting in photosynthesis that is shown in Fig. 5 (for the sake of simplicity the possible existence of a break point phenomenon for the reaction $Y_Z^{ox}S_3 \rightarrow Y_ZS_0 + O_2 + n_3H^+$ is omitted, for discussion, see [112]).

Analyses of the kinetic data within the framework of the Marcus theory of nonadiabatic electron transfer (NET) (for a review, see [46]) and an empirical rate distance relationship of NET [137] leads to two mechanistically important conclusions: (a) the reorganization energies are 0.65–0.75 eV for the oxidation of S_0 and S_1, much larger (about 1.6 eV) for the $S_2 \rightarrow S_3$ transition and values of 1.2–1.4 eV appear to be realistic for the reaction of Y_Z^{ox} with S_3 [75], and (b) values of >15 Å are obtained for the edge-to-edge distances between Y_Z and Mn_4O_XCa [131] that are entirely inconsistent with the known distances from XRDC analyses of Ferreira et. al. and Loll et al. [44,45]. This striking discrepancy is a clear indication that the S_i-state transitions cannot be kinetically limited by NET but are rather reactions triggered by either proton rearrangement or conformational changes.

5.3. Kinetic H/D isotope effect

The oxidation steps of the WOC are characterized by comparatively small kinetic isotope effects (KIEs) of the exchangeable protons with $k_i(H)/k_i(D)$ ratios of 1.3–1.4 (for $i=1$–3) in PS II membrane fragments [131,138,139] and slightly higher values of 1.5–2.5 in PS II core complexes from spinach [131,140]. Interestingly, very similar numbers (about 1.4 for two redox steps leading to O_2 reduction under formation of the "F-state" and about 2.5 for the subsequent transition into the "H(O) state") have been found in cytochrome *c* oxidase [141,142], which catalyzes the reverse reaction, i.e. O_2 reduction to water (for a review, see [143]). These KIE values exclude a break of O–H or N–H bonds as rate limiting steps in the WOC and rather reflect interactions between redox intermediates and protons of basic group(s) of the protein as discussed for cytochrome *c* oxidase [141,142].

6. Mechanism of oxidative water splitting

Fig. 6 presents a proposal for the mechanism of oxidative water splitting in photosynthesis. Consensus exists on the nature of the $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_2$ transitions of the WOC as metal centered reactions but at present an unambiguous assignment to individual manganese ions of the Mn_4O_XCa cluster is not possible (for a detailed discussion, see [78]). Much less information is available for the subsequent oxidation steps of the WOC. The transition $Y_Z^{ox}S_2 \rightarrow Y_ZS_3 + H^+$ involves either a metal-centered reaction (pathway a) or a ligand-oxidation leading to $M_2(LW)_1$ (pathway b) or resulting in a multistate system of S_3, including fast redox isomerism and proton tautomerism (reactions K_{32}^{red}, K_{21}^{red}). Molecular oxygen is only formed after reaching the formal redox state S_4. The nature of this kinetically elusive S_4 of the Kok-cycle (see Fig. 1) is a matter of controversial debate. In order to avoid semantic problems, a clear distinction between the different forms of states $Y_Z^{ox}S_3$ and Y_ZS_4 is indispensable (see Fig. 6). Both states are characterized by the same overall redox level, i.e. the accumulation of four oxidizing redox equivalents compared to state Y_ZS_0. According to the XRDC data [44,45] the redox

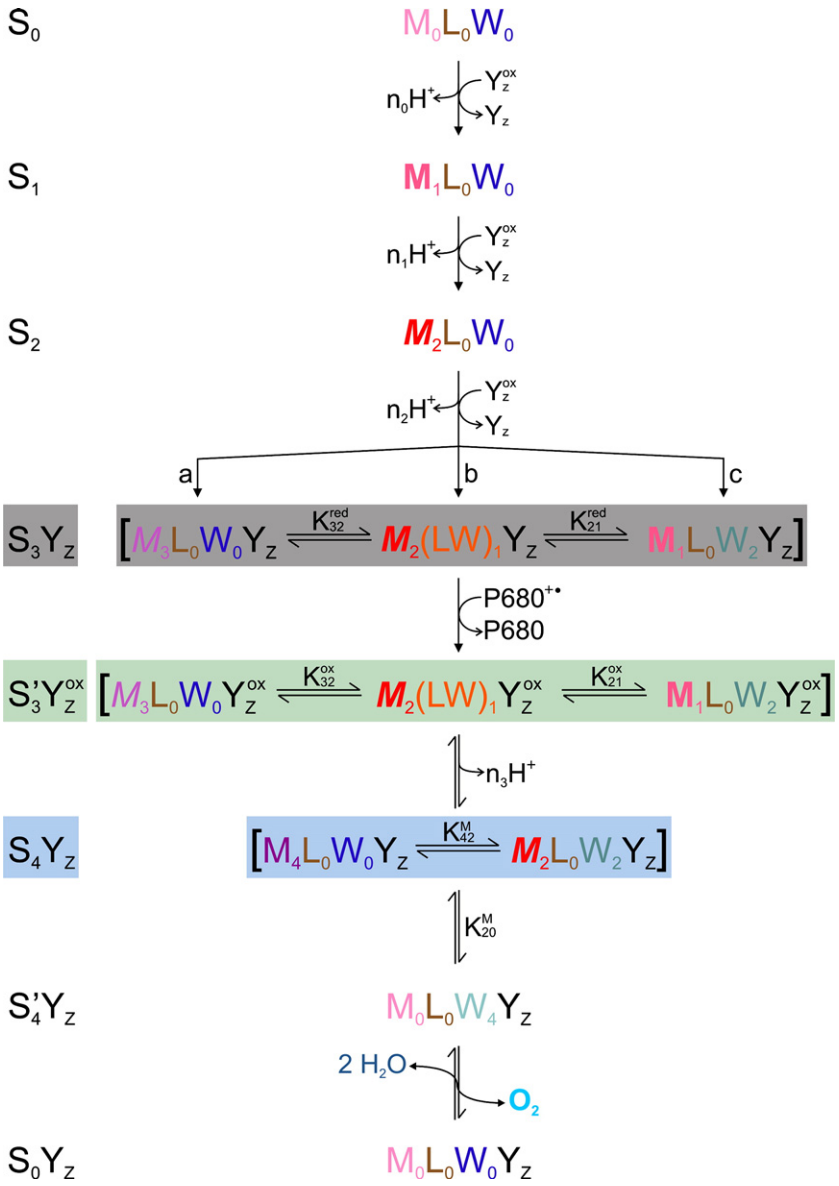

Fig. 6. Simplified mechanism of oxidative water cleavage. The redox states S_i of the WOC are symbolized by $M_jL_kW_l/M_j(LW)_n$ as outlined in the text. Different redox states of the manganese are distinguished by using different colors and types of capital letters M; a, b, c are rate constants for the oxidation of S_2 by Y_Z^{OX} via different pathways; K_{32}^{red}, K_{21}^{red} and K_{32}^{OX}, K_{21}^{OX} are the overall equilibrium constants between the three different states $M_3L_0W_0$, $M_2(LW)_1$ and $M_1L_0W_2$ of S_3 and of $Y_Z^{OX}S_3$, respectively, including redox isomerism and proton tautomerism; K_{42}^{M} and K_{20}^{M} are the equilibrium constants for the two possible states of S_4 and between S_4 and $S_4^{'}$(complexed dioxygen), respectively. The postulated multistate forms, $S_3^{'}Y_Z^{OX}$ and S_4Y_Z are labelled by a light green and blue, respectively, background. Oxidation of Y_Z by $P680^{+\bullet}$ is explicitly shown only for the transition S_3Y_Z $P680^{+\bullet} \rightarrow S_3Y_Z^{OX}$ P680, otherwise only oxidation of the WOC by Y_Z^{OX} is presented.

active component Y_Z is more than 5 Å apart from Mn_4O_XCa and not part of its first coordination sphere while the substrate is most likely directly bound to metal centers. Therefore it seems reasonable to exclude Y_Z^{OX} from the definition of the S_i-states by $M_jL_kW_l$ (see Section 4.2) and to consider $Y_Z^{ox}S_3$ not as an S_4 state. It must be emphasized that this definition is somewhat arbitrary because Y_Z^{OX} is a constituent of the WOC as a holistic system (see below).

The key step of the overall mechanism of oxidative water splitting is the formation of the O–O bond. Two basically different proposals are currently discussed in the literature: (i) the O–O bond is only formed at the redox level S_4 of the WOC or (ii) O–O bond linkage occurs already in S_3 at the level of a complexed peroxide ($M_1L_0W_2$). Two different modes of reaction have been proposed for case (i): (a) nucleophilic attack of a free or loosely bound substrate water molecule onto a highly electron deficient Mn(V)=O group in the S_4 configuration $M_4L_0W_0$ (pathway a, see [144,145]) or (b) radical mechanism (pathway b) where a μ-oxo-bridge radical (state $M_2(LW)_1$) reacts either with another oxo radical built in S_4 or migrates within Mn_4O_XCa to a terminal site and oxidatively interacts with a second substrate water molecule. Both processes lead to formation of the same transient state $M_2L_0W_2$ [146,147]. Results of

recent XANES measurements are not in favour with the nucleophilic attack mechanism (pathway a) [133,148]. On the other hand, a mechanism via pathway b is not in contradiction with the XANES data (for further details see [78]) The preference of an oxygen radical ligand rather than Mn(V)=O type mechanism is supported by recent density functional theory (DFT) calculations [149].

In contrast to the "S_4 dogma", the second alternative (ii) proposes that the O–O bond is formed as a binuclearly complexed peroxide that is already populated in S_3 (form $M_1L_0W_2$)) when Y_Z is still reduced [121]. The key of this model is the postulate that S_3 is a multiple state redox level of the WOC (see Fig. 6) where $M_1L_0W_2$ is one state within both, a rapid redox isomerism equilibrium [120] and a very fast oxy-water↔hydrogen peroxide tautomerism [110,150]. Furthermore, the form $M_1L_0W_2$ of S_3 is assumed to represent the "entatic state" (for details on "entatic state", see [151]) for photosynthetic O_2 formation [110,150]. Oxidation of $M_1L_0W_2$ by Y_Z^{ox} readily leads to formation of $M_2L_0W_2$, i.e. a state that formally corresponds with a peroxide complexed at Mn_4O_xCa in redox state S_2. In all models discussed here state $M_2L_0W_2$ is rapidly transferred via internal redox isomerism and proton shifts into state $M_0L_0W_4$, which represents molecular oxygen complexed at Mn_4O_xCa in state S_0 ($M_0L_0(O_2)_{complexed}$), followed by eventual product/substrate exchange under the release of molecular oxygen. Clausen and Junge [122] found that at increasing O_2 pressures (range of 10–20 bars) reduction of Y_Z^{ox} is suppressed after excitation of dark adapted PS II core complexes with the 3rd flash of a sequence of single turnover flashes and interpreted these data by the existence of equilibria between states $Y_Z^{ox}S_3$, Y_Z S_2 $(H_2O_2)_{complexed}$ and $Y_ZS_0+O_2$.

The scheme of Fig. 6 does not include mechanistic considerations on the protolytic reactions because our knowledge on this topic is rather fragmentary. Regardless of the missing information, however, it is clear that the local proton activity and the existence of proton gradients $\nabla H^+(\vec{r},t,S_i)$ near the catalytic site are of special mechanistic relevance. $\nabla H^+(\vec{r},t,S_3)$ is assumed to be a key parameter for the O–O bond formation in S_3 [118]. The nature of this gradient is determined by the properties of protonatable groups from the local protein environment of the Mn_4O_xCa cluster. Therefore parts of the protein itself actively participate in the catalytic reaction. As a consequence the WOC has to be considered as a molecular machine that is specially tailored for oxidative water splitting [150]. This holistic view of the WOC also comprises pathways for substrate entry and product release [118,152,153].

In summary, the idea of a binuclearly complexed "peroxide" intermediate [110,121] seems to be a realistic model but the mechanistic details still remain to be clarified.

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