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Minireview

The mechanism of photosynthetic water oxidation

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Abstract. Photosynthetic water oxidation is unique to plants and cyanobacteria, it occurs in thylakoid membranes. The components associated with this process include: a reaction center polypeptide, having a molecular weight (Mr) of 47–50 kilodaltons (kDa), containing a reaction center chlorophyll *a* labeled as P680, a plastoquinol(?)-electron donor Z, a primary electron acceptor pheophytin, and a quinone electron acceptor Q_A; three 'extrinsic' polypeptides having Mr of approximately 17 kDa, 23 kDa, and 33 kDa; and, in all likelihood, an approximately 34 kDa 'intrinsic' polypeptide associated with manganese (Mn) atoms. In addition, chloride and calcium ions appear to be essential components for water oxidation. Photons, absorbed by the so-called photosystem II, provide the necessary energy for the chemical oxidation-reduction at P680; the oxidized P680 ($P680^+$), then, oxidizes Z, which then oxidizes the water-manganese system contained, perhaps, in a protein matrix. The oxidation of water, leading to O_2 evolution and H^+ release, requires four such independent acts, i.e., there is a charge accumulating device (the so-called S-states). In this minireview, we have presented our current understanding of the reaction center P680, the chemical nature of Z, a possible working model for water oxidation, and the possible roles of manganese atoms, chloride ions, and the various polypeptides, mentioned above. A comparison with cytochrome *c* oxidase, which is involved in the opposite process of the reduction of O_2 to H_2O, is stressed.

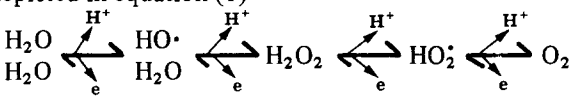
This minireview is a prelude to the several minireviews, scheduled to be published in the forthcoming issues of *Photosynthesis Research*, including those on photosystem II (by H.J. van Gorkom); polypeptides of the O_2-evolving system (by D.F. Ghanotakis and C.F. Yocum); and the role of chloride in O_2 evolution (by S. Izawa).

I. Introduction

The mechanistic realization of photosynthetic water cleavage by visible light is the cornerstone in the evolutionary development for the exploitation of solar radiation as a free energy source for the biosphere. The 'invention' of this reaction about 2–3 billion years ago, realized in cyanobacteria, not only made the huge water pool on the earth's surface available as an electron source for the generation of metabolically bound hydrogen, but in addition enriched the anaerobic atmosphere with oxygen up to the present level. In this way, oxygen became available as a unique oxidant for extensive

free energy release from food, allowing the development of highly organized heterotrophic organisms [for a recent review see ref. 1]. Accordingly, the 'handling' of the redox couple H_2O/O_2 in biological organisms is of crucial importance and is the focus of current research activities in several laboratories.

If one considers the sequence of univalent redox steps between H_2O and O_2 as depicted in equation (1)



(1)

it is easily seen that the intermediates are very harmful species for biological systems. Therefore, the essential problem to be solved by nature was the development of enzyme complexes that catalyze the reaction sequence of equation (1) without producing harmful species. Two key enzyme complexes were 'invented': (a) the cytochrome c-oxidase for the exergonic oxygen reduction to water in the respiratory chain [see e.g. ref. 2], and (b) the water oxidizing (or oxygen evolving) enzyme system [see e.g. ref. 3] in order to catalyze oxygen formation from water by sufficiently oxidizing redox equivalents produced via photoreaction within the so-called reaction center complex of photosystem II (PS II) (referred to as RC II). Despite the fact that both enzyme complexes are of great interest for the understanding of the principles required for the performance of reaction sequence (1) in biological organisms, the present discussion will be restricted to the water oxidizing enzyme system. We shall, however, make a few additional comments on the reaction center complex RC II as well as on the functional coupling between RC II and the water oxidizing enzyme system.

II. The reaction center complex RC II

In green plants there are two different photosystems (I and II) [see e.g. ref. 4]. Photosystem I (PS I) produces a strong reductant (NADPH, reduced form of nicotinamide adenine dinucleotide phosphate) and a weak oxidant (plastoquinone, PQ); whereas, PS II reduces PQ to PQH_2 and generates a strong oxidant required for oxidation of water to O_2. Details of PS II and PS I will be covered in other minireviews, in *Photosynthesis Research,* by H.J. van Gorkom, and A. W. Rutherford, respectively. The PS II reaction is initiated by light absorption in the pigment complexes of PS II, followed by excitation energy transfer to RC II where the primary photochemical reaction takes place.

During the last decade the general organization scheme of RC II has been unraveled; it resembles that of photosynthetic purple bacteria [for a recent review see ref. 5], but almost all mechanistic details still remain to be clarified. The crucial functional difference with the reaction center of purple bacteria is the necessity for RC II to produce redox equivalents of a free energy

that is sufficient for water oxidation. This goal is achieved using a specifically bound chlorophyll-*a*, referred to as P680, as the photoreactive pigment (P stands for pigment and 680 refers to the absorption maximum wavelength of its lowest electronic transition). It is not yet clear whether P680 is a mononomeric chlorophyll-*a* molecule [6] or a dimeric chlorophyll-*a* aggregate [7]. $P680^*$ ejects an electron from its lowest excited singlet state within a few ps (according to latest estimations from fluorescence lifetime measurements, the reaction time τ_p is $\leqslant 1$ ps [see ref. 8]). This electron is transferred in less than 1 ns over a distance of 2–3 nm to a special plastoquinone complex with an iron center (this iron associated PQ is designated as Q_A). The reaction involves at least one redox intermediate identified as pheophytin [see e.g. 9]. Recent experimental data favor the existence of additional redox intermediates [10, 11, 12]. Charge stabilization sufficient for water oxidation is achieved only if the electron from P680 can be accepted by Q_A [13]. As the RC II complex is anisotropically incorporated into the thylakoid membrane, electron transfer from $P680^*$ to Q_A generates, simultaneously, an electrical potential difference [14].

The kinetics of the electron transfer steps have not been measured directly. However, regardless of the unresolved details, the quantum yield of the overall charge separation within RC II is close to one [15]; this requires a delicate balance of the rate constants for the forward and back reaction of each individual electron transfer step [16]. This essential regulatory control is easily achieved since the functional redox groups are embedded into a specific protein matrix reported to have a molecular weight of 47–50 kDa [17, 18]. Interestingly enough, all redox components in RC II identified so far are π-electron systems. Accordingly, the RC II photo-redox-chemistry can be considered to be a very specialized vectorial intermolecular π-electron shift regulated by the RC II-apoprotein.

The mechanistic details are not yet understood. In vitro studies have revealed the importance of point charges in the microenvironment of porphyrins [19] for their spectral and reactive properties and the functional role of the mutual orientation of reactive redox groups [20, 21]. These few data are consistent with the idea that the RC II-protein matrix is of great importance for determining the rate constants as well as the redox properties (especially of the $P680^*/P680^+$ couple). Therefore, we shall pay special attention in our future research to the studies on the functional role of the 47–50 kDa PC II polypeptide. Regarding the stabilization of electron transfer via a connected π-electron system, a key step for modeling photosynthetic reaction centers has been achieved through the synthesis of a system containing tetraarylporphyrin with covalently bound quinone and carotenoid moieties [22].

III. The basic organization scheme of photosynthetic water oxidation

The basic principles of the functional organization scheme of photosynthetic

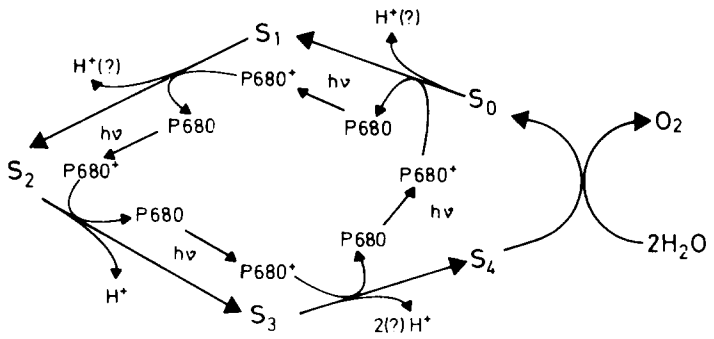


Figure 1. Simplified scheme of the redox cycle leading to photosynthetic water oxidation (for details see text).

water oxidation became apparent 15 years ago after the classical polarographic experiments of P. Joliot and coworkers and of B. Kok and coworkers. They revealed a characteristic oscillation pattern of oxygen evolution [for a review, see ref. 3, or, 23]: the maxima (and minima) of the oxygen yield followed a periodicity of 4 with flash number, when dark adapted samples were exposed to a train of saturating light flashes (flash duration a few μs, time between flashes 0.3–1 s). The oscillations are damped with increasing flash number. Unexpectedly, the first maximum of oxygen yield occurred after the third flash. Kok and coworkers [24] presented a scheme (Figure 1) in which the water oxidizing enzyme system becomes sequentially oxidized with $P680^+$ as ultimate oxidant by univalent redox steps (the redox states are labeled S_0, S_1, S_2, S_3 and S_4) until after accumulation of four oxidizing equivalents (S_4) oxygen is evolved. In order to match the experimental details of the oscillation pattern it was assumed that the dark-adapted system starts with 25% S_0 and 75% S_1 (some data favor an almost 100% S_1 population [25]) and that each redox transition includes a certain possibility of misses (no transitions) and double hits (double transitions). The above-mentioned data and other findings also imply the absence of large scale cooperation among different reaction center complexes and the water oxidizing enzyme system. The lifetime of the last redox state in the sequence (Figure 1), S_4, is very short ($\leqslant 1$ ms), the precursor states S_2 and S_3 are stable for several tens of seconds, whereas transitions between S_0 and S_1 are very slow (order of 30 min) with S_1 appearing most stable [25] under normal conditions in the dark [for a review about lifetime of S_i-states, see ref. 23 and recent data in ref. 25]. An interesting observation, made by Lavorel and Maison-Peteri [25a], is that the state S_2 is most abundant and $[S_1] + [S_3] =$ constant at steady state over a broad range of non-saturating flash intensities, in the green alga *Chlorella pyrenoidosa*. Furthermore, these observations are best explained by proposing the existence of a charge carrier (C) that stores two positive charges as S_3 deactivates to S_1; and that these charges are conserved in the transition of S_0 to S_2. The existence

of a charge carrier also explains the high quantum yield of O_2 evolution at low light intensities in the face of deactivation of higher S-states.

Based upon EPR measurements, the oxidation kinetics of the water oxidizing enzyme system were inferred to be dependent on its redox state S_i, characterized by half times of 0.1–1 ms [26]. These reactions are much slower than the $P680^+$ reduction detected by absorption changes at 820 nm [27, 28] and 690 nm [29]. Accordingly, P680 and the water oxidizing enzyme system are connected by at least one redox component, referred to as Z (or D_1). This raises questions about the nature of Z. However, the more interesting question about the mechanism of photosynthetic water oxidation is the characterization of the functional redox groups of the S_i-states and their kinetic regulation by the protein matrix of the water oxidizing enzyme system.

IV. The nature of component Z

Analyses of EPR spectra have led to the conclusion that the functional redox group of Z is a special plastoquinol (PQH_2) that functions as a one-electron carrier with PQH_2^+, as cation radical, acting as the oxidizing species [30]. This assignment is roughly consistent with latest reports on the absorption difference spectra assigned to the Z^{ox}/Z couple [31–33]. The stabilization of PQH_2^+ (in Tris-washed chloroplasts, Z^{ox} is reduced via multiphasic kinetics in the ms to s range) [see ref. 33] requires the existence of a very special protein matrix, because the pK-value of PQH_2^+ in aqueous (50%) ethanolic solutions is reported to be negative [34]. Accordingly, immediate deprotonation would arise without a protective microenvironment. The Z-oxidation was found to be coupled with a stoichiometric proton release [35, 36] that could be due to deprotonation of either the functional redox group of Z^{ox} or of the surrounding protein moiety (Bohr-effect). Therefore, these data do not permit an unambiguous identification of the protonation state of the functional redox group in Z^{ox}. In addition to the evidence for PQH_2^+ based on the EPR spectrum of Z^{ox}, it has been argued that the cation radical is required for sustaining sufficient oxidizing power for the S_i-state transition in the water oxidizing enzyme system. This argument, however, is not convincing because numerous enzymes are known whose apoproteins markedly affect the redox potential of the functional group. However, regardless of the details of the protonation, the idea that the functional redox group of Z is a special plastoquinol necessarily implies the existence of a very specific apoenzyme which either prevents proton escape from PQH_2^+ or significantly enhances the oxidizing power of a plastosemiquinone radical (PQH°).

The kinetics of electron transfer from Z to $P680^+$ become markedly slower after selective elimination of the oxygen evolving capacity [37]. This effect might be ascribed to a redox potential shift of Z as discussed

previously [38].Recently, it has been estimated that, after Tris-washing (this procedure destroys the oxygen evolving capacity), the redox potential of Z^{ox}/Z is decreased more than 120 mV [39]. This finding might be the first piece of evidence for redox potential regulation by the protein matrix of Z or by other regulatory polypeptides at the donor side of PS II (vide infra). At the writing of this review, Z has not been identified unambiguously, and, especially, information about the protein matrix is lacking.

V. A new ESR signal

Casey and Sauer [39a] and Zimmerman and Rutherford [39b] have reported a new ESR signal at $g = 4.1$ with a linewidth of 360 Gauss. This signal was attributed to a precursor of one of the S-states (S_2) of the water oxidation system [39a]. It is formed by illumination at 200 K, not by flash excitation at room temperature; it does not occur in the presence of diuron (which blocks electron flow out of Q_A^-); it is absent in Tris-washed membranes; and it has been suggested to arise from an intermediate donor (let us call it Z_2) between the S-states and Z (let us call it Z_1) [39b]. Further research is needed to understand its role in photosynthetic water oxidation.

VI. The water oxidizing enzyme system

The central problem of water oxidation via a four-step univalent redox sequence, within biological organisms containing very sensitive components, is how to stabilize the reactive intermediates in order to prevent rapid oxidative destruction. Generally speaking, this goal can be achieved by using appropriate redox groups which are intercalated into a specific protein matrix acting as apoenzyme. An inspection of the enzymology of oxygen [40] reveals that the functional redox groups are either organic π-electron systems (flavins, etc.) or transition metal centers (Fe, Cu, Mn, Mo). It has been known for a long time that photosynthetic water oxidation indispensibly requires manganese [for reviews, see refs. 3, 41, and 42]. Accordingly, it appears logical to consider the water oxidizing enzyme system as basically a manganese protein with metal centers associated with a specific coordination shell acting as functional redox groups [43, 44]. Based upon this assignment, the following questions have to be answered: (a) What is the structural arrangement and valence of the manganese in the different S_i states of this system? (b) What is the nature of the coordination shell, including water and its redox intermediates as likely ligands? (c) What is the polypeptide pattern of this system? (d) At which redox states are protons released?

VI. 1 The functional role of manganese

There are at least three different pools of bound manganese but only one of them was inferred to be directly involved in water oxidation [3, 41, 42].

Despite extensive studies, the exact number of functional manganese ions (or atoms) has not yet been determined unambiguously. Based upon treatment with hydroxylamine or Tris-washing which cause destruction of the oxygen-evolving capacity, accompanied by displacement of manganese ions (or atoms) from its native sites, a number of four manganese ions (or atoms) per water oxidizing enzyme system was obtained [3, 41, 42]. However, two of the four manganese ions (or atoms) were found to be different with regard to their magnetic interaction with the oxidized donor, Z^{ox} [45]. Based on the fact that partial release of manganese ions (or atoms) after Tris-washing of inside-out thylakoids did not prevent reconstitution of the oxygen-evolving capacity, only two manganese atoms (or ions) were inferred to be directly involved in water oxidation [46]. Similar conclusions were made after quantitative measurements of the manganese content of PS II particles [47]. However, it should be emphasized that these data do not provide unambiguous evidence that the functional center of water oxidation contains only two manganese. A tetrameric cluster as functional unit cannot be excluded (vide infra).

The valence states of the manganese have not yet been resolved. The functional manganese in situ appears to be EPR silent at room temperature, but after destruction of water oxidizing enzyme system an EPR spectrum typical for $[Mn(H_2O)_6]^{2+}$ can be observed [3, 41, 42]. This result reveals that manganese is either surrounded by a specific ligand shell or that it attains an EPR-inactive valence state. An approach to this problem was the quantitative determination of Mn(II)-release after mild heat shock treatment [48]. The results obtained were interpreted by a model with a binuclear manganese complex which attains the valence state (Mn(II)-Mn(II) in S_0 of the water oxidizing enzyme system. During the redox cycle, the Mn centers were assumed to reach maximally the Mn(III) state in S_1, S_2, S_3 and S_4 (a transitory state S_2^* on the route from S_2 to S_3 was inferred to include Mn(IV)). Further support for a mixture of Mn(II) and Mn(III) states in this system was provided by XAES investigations [49]. Attempts to use the effect of Mn^{2+} on the proton nuclear spin relaxation [50] have not provided unambiguous results [for latest data, see ref. 51].

New inroads into the complex problem of the manganese valence states were opened by the discovery of a multiline EPR signal for Mn at very low temperatures (< 15 K), which was ascribed to the state S_2 [52–54]. The amplitude of this signal oscillates with a period of four, with maxima on the first and the fifth flashes [39b, 52]. Unfortunately, the results do not yet permit an unambiguous conclusion about the structure of the functional manganese group [52, 53]. However, regardless of the controversy about the existence of a binuclear [53] or a tetrameric cluster [52], the results favor the idea that Mn(III) is the predominant valence state and that Mn(II) occurs only at one manganese atom in state S_0. Furthermore, one manganese atom in the S_2-redox state is inferred to attain Mn(IV) valency.

This very brief summary of the pertinent results shows that the valence state of manganese in situ still remains an unresolved problem, but the different results provide ample indirect evidence for direct participation of manganese in the redox cycle leading from water to molecular oxygen. The above-mentioned results are also interpreted to show that the electron abstraction during the $S_i \rightarrow S_{i+1}$ transition is not only restricted to the manganese centers but that ligand groups are also involved. This raises questions about the nature of these redox active ligands. In the simplest case, water itself (and its different redox intermediates) as inner sphere ligand functions directly as electron donor. However, participation of other ligands cannot be excluded. Accordingly, an answer to the question whether or not water is the only redox active ligand of the manganese center is of crucial importance for understanding the mechanism of photosynthetic water oxidation.

VI. 2 Mechanism of photosynthetic water oxidation

Generally, three different models are considered: (a) the redox reactions are restricted to the manganese-water system (it will be referred to as Mn-H_2O-model); (b) in addition to Mn and H_2O another ligand or component is involved in the redox cycle (L-Mn-H_2O-model); and (c) the redox steps occur mainly via the non-H_2O ligand system (Mn-L-H_2O-model).

Information about the participation of water in the intermediary redox steps could be obtained by measuring the proton release pattern coupled with the sequence of S_i state transitions (see Figure 1). The stoichiometric details of H^+-liberation are not completely solved. At this time a 1:0:1:2 sequence during $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$, $S_3 \rightarrow S_0$ transitions appears to be favored [55, 56a], although some other findings support a 0:1:1:2 stoichiometry [57, 58]. The apparent proton release pattern depends on the mode of dark adaptation [59] The water oxidizing enzyme was shown to become inactivated in long dark adaptation, but a 2 min dark adaptation, preceded by illumination, provides an active system [60]. Thus, the above measurements should be repeated under strictly controlled dark adaptation conditions. Latest refined kinetic studies favor a 1:0:1:2 pattern with half times of 250 μs, 200 μs and 1.2 ms, respectively (V. Förster and W. Junge, personal communication). A complication in the interpretation of the experiment is the possibility of proton binding by intrinsic groups (e.g., aminoacid residues) which do not equilibrate with the external medium. In this case the 'intrinsic' deprotonation pattern of water oxidation does not correspond to the extrinsically measured proton release, as discussed previously [61]. Regardless of these quantitative problems, the reported data highly support the idea that water is directly involved as redox substrate and participates earlier than in the last step of the sequence [62, 63].

Before discussing further details of the above-mentioned models, it should

be emphasized that in any case a further crucial point has to be considered, i.e., the water oxidizing enzyme system contains one redox equivalent under normal, dark adapted conditions. In order to account for the experimental findings so far, a detailed model of the L-Mn-H_2O-type has been proposed [for reviews, see refs. 64 and 65] which involves the following *postulates:* (a) Water oxidation occurs at a binuclear manganese cluster with water as inner sphere ligand. The intermediary redox states formally corresponding to $HO^{\bullet}$ and H_2O_2 are highly stabilized (80–120 kJ/mol, compared to the corresponding free species) by electron delocalization between the manganese centers and the ligand. (b) The $S_0 \rightarrow S_1$ transition represents the oxidation of a component M which is only moderately oxidizing in its M^{ox} state. It could act as bridging ligand, L_B, of the binuclear manganese cluster. (c) Dioxygen-bond formation occurs at the peroxide redox level as a binuclearly complexed hydrogen peroxide. (d) Binuclearly complexed superoxide readily reacts with M^{ox} to form complexed oxygen so that practically a two-electron transition leads from peroxide to oxygen (an analogous two-electron transition leading in the opposite direction from O_2 to H_2O_2 in cytochrome oxidase has been reported, [see ref. 66, 67]. (e) Oxygen is released via an exergonic exchange reaction with the two water molecules.

This model implies a reaction mechanism which is described by equations (2)–(6), (for the sake of simplicity the manganese centers are omitted, one and two asterisks represent mono- and binuclear complexation, respectively; and the kinetic data were taken from a recent report [68]):

$$[(H_2O)_2^*]\,M \xrightarrow[50\,\mu s]{\oplus} [(H_2O)_2^*]\,M^{ox} \qquad (\equiv S_1) \quad (2)$$

$$[(H_2O)_2^*]\,M^{ox} \xrightarrow[50\,\mu s]{\oplus} [(H_2O)^*(OH)^*]\,M^{ox} + H^+ \qquad (\equiv S_2) \quad (3)$$

$$[(H_2O)^*(OH)^*]\,M^{ox} \xrightarrow[1\ ms]{\oplus} [(H_2O_2)^{**}]\,M^{ox} + H^+ \qquad (\equiv S_3) \quad (4)$$

$$[(H_2O_2)^{**}]\,M^{ox} \xrightarrow[1\ ms]{\oplus} [(O_2^- \ldots H^+)^{**} + H^+]\,M^{ox}$$

$$\xrightarrow{k_b} [(O)_2^{**}]\,M + 2H^+ \qquad (\equiv S_4) \quad (5)$$

$$[(O_2)^{**}]\,M + 2H_2O \xrightarrow{k_{O_2}} [(H_2O)_2^*]\,M + O_2 \qquad (\equiv S_0) \quad (6)$$

The above model predicts an intrinsic deprotonation stoichiometry of either 1:1:1:1 or 0:1:1:2, depending on the properties of component M (in equations (2)–(6) M-oxidation is assumed not to be coupled with H^+-release). No restrictions are made about the valence state of manganese, but in the redox state S_2 the metal centers within the functional binuclear manganese group are inferred to be likely in a higher oxidation state compared to S_3 due to different degrees of electron delocalization between central ion and oxidized water ligand for $(OH)^*$ and $(H_2O_2)^{**}$, respectively [62].

The actual valence state of the manganese depends strongly on the nature of the overall coordination sphere. Investigation of synthetic manganese complexes shows that the redox potentials for the transitions Mn(II) ⇌ Mn(III) ⇌ Mn(IV) can vary over a wide range for different ligand derivatives [69, 70]. Therefore, no statements about the electronic configuration of the manganese centers can be made unless detailed experimental data are available, but it should be mentioned that recent low temperature EPR data have been interpreted in terms of Mn(III) for state S_1 [53, 71]. However, it should be kept in mind that low temperature measurements may not reflect the electronic situation in vivo, because at room temperature the distribution of electron density between ligand and central ion could be different, based on recent reports on model compounds [72, 73]. Component M is not specified by the model. It could be either a bridging ligand (e.g., a special plastoquinone) or a separate component or even only a symbol for a unique manganese valence state in the oxygen evolving system (see below). Regardless of these details, the $S_0 \rightarrow S_1$ transition is assumed not to change the redox state of the water ligand.

The different behavior of S_1 compared to S_2 and S_3 is evident not only from the quite different lifetimes [for reviews, see refs. 23, 25 and 41] but is also manifested by the sensitivity of the latter states to different substances, such as ADRY* agents [74] or amines [75]. The water oxidizing enzyme system is very sensitive to high pH [76] and Tris-treatment [75] in redox state S_2. This result supports the idea of a special manganese oxidation in S_2. The proposed model also implies a special energetic pattern for water oxidation via a four-step univalent redox sequence [for details see refs. 61, 62]. For example, the redox transitions between $(H_2O)^*$ and $(OH)^*$ corresponding with $S_1 \rightarrow S_2$ and between $(OH)^*$ and $(H_2O_2)^{**}$ reflecting $S_2 \rightarrow S_3$ are assumed to require practically the same free energy. This assumption has been confirmed by thermodynamic calculations (based on data of delayed fluorescence and back reaction kinetics) [see ref. 77] and by thermoluminescence measurements [78].

A very important aspect of the mechanism of water oxidation is the structural dynamics coupled with the S-state transitions, especially as a consequence of dioxygen formation. A first hint for structural changes might be the S_i-state dependent inhibition of oxygen evolution by p-nitrothiophenol [79]. The S_i-state dependent electron transfer kinetics of $P680^+$-reduction [for indirect evidence, see refs. 38, 80] and of the S_i-state advancement (for direct EPR-measurements of Z^{ox}-reduction, see refs. 26, 68) might also be interpreted in terms of redox transition induced conformational changes in the water oxidizing enzyme system. The marked differences in Z^{ox}-reduction kinetics coupled with transitions $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$ and $S_2 \rightarrow S_3$, $S_3 \rightarrow (S_4) \xrightarrow{2H_2O} S_0 + O_2$ might reflect significant structural

* ADRY stands for *a*cceleration of *d*eactivation *r*eactions of the system *Y*, where Y symbolizes the water oxidizing enzyme system.

changes during dioxygen bond formation at the redox level S_2. Conformational changes in redox enzymes have been well established [81] but no detailed studies are known for the water oxidizing enzyme system in relation to the redox reactions. Another important fact is the conformational relaxation of the apoenzyme after a redox transition of the functional metal centers. The role of this structural dynamics for the overall mechanism of photosynthetic water oxidation is completely unresolved.

Other models of the L-Mn-H_2O-type have been proposed referring either to the characterization of S-state transition patterns [82] or to postulation of plastoquinone as an essential manganese ligand that participates in the redox sequence [73, 83]. Because in manganese quinone-complexes, the ligand modulates the reversible O_2-binding capacity [84] one might speculate about the regulation of the exergonic O_2-H_2O-water exchange (equation (6)) by a quinone ligand. Extending these studies on manganese-3,5 di-tert.-butyl quinone complexes and taking into account mechanistic studies about the catalytic function of μ-oxobridged Ru(III)-complexes [84], a model has been recently proposed [73] for photosynthetic water oxidation that includes two fundamental steps: (a) conversion of two water molecules into adjacent oxo-ligands at a binuclear manganese center, coupled with Mn(II) → Mn(IV) transition and deprotonation of the ligands; and (b) O_2-formation by displacement of the two oxo-ligands, probably supported by manganese reduction to Mn(II). This could give rise to destabilization of the oxo-ligands, that are finally oxidized to O_2 by the catecholat-form of the ligands. Accordingly, redox transitions at the manganese as well as at the quinone ligands and the two adjacent oxo-ligands participate in water oxidation. The model in the present form, however, has to be modified significantly in order to explain the specific patterns of oxygen evolution and proton release of photosynthetic water oxidation.

Another possible role of quinone could be the function as a bridging ligand between two manganese centers, thereby simultaneously providing their magnetic coupling, as has been inferred by studies on model complexes [72; also see 85]. In this case, however, the quinone ligand is not involved in the redox reactions. It is suggested that the $S_0 \rightarrow S_1$ transition also represents a manganese oxidation step. So far, no direct experimental evidence for the participation of a quinone in the water oxidizing enzyme system has been obtained. The requirement for artificial PS II-donors for sustaining the linear electron transport in chloroplasts, mildly depleted of plastoquinone [86], might support the above mentioned ideas on a plastoquinone function. This result, however, cannot be considered as unambiguous evidence, because lipid extraction could primarily remove the functional group of Z, as a likely plastoquinone target, rather than modify the water oxidizing enzyme system itself.

Most of the models developed on the basis of EPR measurements are of the Mn-H_2O-type, i.e., redox reactions are restricted to manganese and its

associated water ligand. The essential features of two recently proposed models [53, 71] are very similar to the previously discussed [61, 62, 64] L-Mn-H_2O-type model with the specific assignment of the S_0-state to a functional group with one manganese in the Mn(II) valence state. In all other states manganese is in Mn(III) and/or Mn(IV)-state. This type of model has been theoretically analyzed by quantum mechanical calculations with special emphasis on the structure of the binuclear manganese cluster and its water ligand [87]. All models reported so far consider water in the inner coordination sphere as the essential redox active species. A different situation, however, arises for the Mn-L-H_2O-type models, where the main redox steps occur at a specific ligand. An interesting chemical model assumes that the redox sequence occurs at a chlorine ring system including the intermediary formation of a dioxolium species [88]. Another model claims bicarbonate to be the essential manganese ligand for water oxidation [89].

Despite the lack of unambiguous experimental evidence, the basic principles of the model described by equations (2)–(6) seem to be logical with open questions regarding the nature of the component **M** (either a separate component, e.g., a special ligand, or a special valence state of a manganese center) and the real electronic configuration of the manganese centers in the different S_i-states. This conclusion is not only based on the experimental data briefly outlined here, but also by comparison with the mechanisms of other enzymes, which deal with free intermediates of water oxidation such as a catalase or a superoxide dismutase. Of special interest in this regard, however, are the oxidases that catalyze the back reaction of water oxidation (equation (1)). It is known that in cytochrome oxidase as well as in laccase the redox sequence occurs at a binuclear transition metal center [for a review see ref. 40]. Accordingly, it seems reasonable to assume that the same principle of handling the H_2O/O_2-redox system may have been applied by photosynthetic organisms in order to perform water oxidation.

An important question which remains to be clarified by future work is the structure of the coordination sphere of the functional manganese groups. If one compares the water oxidizing enzyme system with the cytochrome oxidase, one might think about the porphyrin ring as a widely used quadridentate ligand. It is also an important ligand of in vitro manganese model systems, e.g., for O_2 activation in oxygenase with manganese undergoing redox transitions between states Mn(III) and Mn(V) [90, 91]. However, so far no experimental evidence has been found for porphyrins acting as manganese ligands in the water oxidizing enzyme system. Furthermore, no other inner sphere ligand of manganese has been experimentally identified as yet.

After firm experimental evidence of its functional role in oxygen evolution [92], chloride has been discussed as a possible ligand in the O_2-evolving system [e.g. ref. 44]. Recent elegant work [for reviews see refs. 93, 94] led to the conclusion that Cl^- is essential for stabilizing the func-

tional manganese center, particularly in state S_2 and it is also required for the $S_1 \rightarrow S_2$ transition. However, based on later measurements of flash-induced chlorophyll-a fluorecence changes (rise kinetics and yield) and delayed light emission, it has been concluded that Cl^- depletion does not affect the $S_1 \rightarrow S_2$ transition but rather prevents further oxidation from S_2 to S_3 [95, 96]. These contradictory conclusions need to be resolved. Based upon ^{35}Cl-NMR-measurements [97, 97a] it was inferred that during the S_i turnover Cl^- unbinding and rebinding takes place in a specific micro-pocket [94]. This phenomenon is assumed to be correlated to the proton release. The molecular mechanism of this effect is not yet clear, but it is not absolutely specific for Cl^-, because other anions (e.g. Br^-) can substitute for Cl^- but with lower efficiency [92–94]. The specificity is suggested to be dependent upon the size of the anion and the ionic field [97]. Furthermore, a comparatively large pool of 10–20 Cl^- per water oxidizing enzyme system has been estimated [94, 98]. Very recent EPR data, indicating a competition of amines as Lewis-bases for Cl^--binding sites and the effect of Cl^--depletion upon the EPR signal assigned to Z^{ox}, led to the conclusion that Cl^- functions as a bridging ligand between manganese centers, thereby also facilitating electron transfer reactions [99]. Obviously, only part of the Cl^--pool can participate as a bridging ligand. Therefore, future studies have to clarify whether or not Cl^- really functions as a bridging ligand of the functional binuclear manganese center and how this effect is related to the other effects proposed for Cl^- (vide supra). A forthcoming review by S. Izawa, in *Photosynthesis Research,* will discuss more fully the role of Cl^- in O_2 evolution.

Bicarbonate is another component which has been discussed as a possible essential component for oxygen evolution [for a review see ref. 100], but it now seems clear that the main HCO_3^- effect, at least in the presence of HCO_2^-, is located on the electron acceptor side of system II [see refs. 94, 101, 102, 102a].

One of the difficulties arising in the identification of functional groups in the water oxidizing enzyme system is the lack of detailed information on their spectral characteristics in the UV/VIS-range. Generally, the characteristic quaternary oscillation of the water oxidizing enzyme system turnover [vide supra, ref. 23] should provide a labeling of absorption changes associated with the S_i-state transitions. Unfortunately, a binary oscillation due to the acceptor side of system II is superimposed on this pattern. However, it has become possible to eliminate this binary oscillation pattern by trypsination of chloroplasts without seriously affecting the turnover of the water oxidizing enzyme system. It was found [103] that the amplitude of absorption changes in the UV characterized by a 1 ms relaxation kinetics (symbolized by $\Delta A_{n,1}^{4\cdot5}$) oscillates synchronously with the oxygen yield. As the rate of Z^{ox}-reduction by the water oxidizing enzyme system in the redox state S_3 [26, 68] practically coincides with that of oxygen release [23],

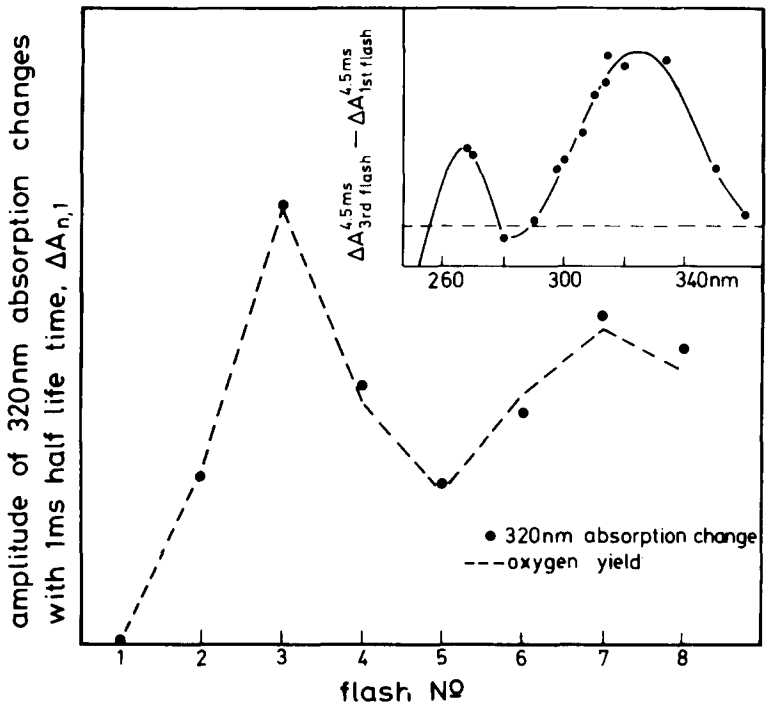


Figure 2. Amplitude of the 1 ms component of flash-induced absorption changes at 320 nm, $\Delta A^{4.5}_{n,1}$, as a function of flash number in dark-adapted trypsinized chloroplasts. The data represent an average of 10 measurements. *Insert:* difference spectrum of the 1 ms kinetics of flash-induced absorption changes reflecting the $Z^{ox}S_3 \rightarrow (ZS_4) \xrightarrow[2\ H_2O]{} ZS_0 + O_2$ transition. [For details, see ref. 103.]

the amplitudes of the 320 nm absorption changes depicted in Figure 2 reflect the turnover of the reaction $Z^{ox}S_3 + 2\ H_2O \rightarrow ZS_0 + O_2$ (also see Figure 3). As neither water nor O_2 absorbs in the range of 250–350 nm, the absorbance difference between the states S_3 and S_0 can be obtained by subtraction of the Z^{ox}/Z-difference spectrum. The difference spectrum of the Z^{ox}/Z-redox couple obtained from measurements of inside-out thylakoids with high oxygen-evolving capacity (W. Weiss and G. Renger, in prep.) depicted in Figure 3 closely resembles that of Tris-washed PS II-particles [31]. Using these data, the difference spectrum ascribed to the absorbances of S_3 versus S_0 is obtained. Figure 3 shows that this difference spectrum is characterized by a rather broad band peaking around 320 nm. A comparison with a manganese-gluconate model system [104] might suggest that the $S_3 \rightarrow S_0$ transition involves an Mn(IV) → Mn(III) redox reaction. However, other interpretations, including the possibility of ligand absorption

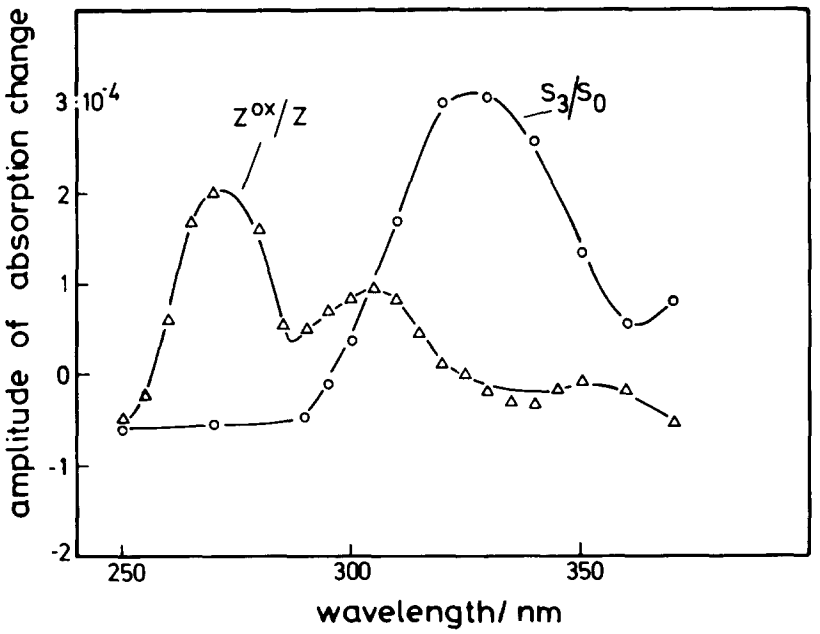


Figure 3. Difference spectra of Z^{ox} versus Z and S_3 versus S_0 calculated from data measured in inside out thylakoids (W. Weiss and G. Renger, in preparation) and in trypsinized normal thylakoids [see ref. 103].

changes, e.g., due to the proposed redox turnover of plastosemiquinones [73, 83], cannot be excluded. A recent, more refined analysis of flash-induced absorption changes measured in chloroplast preparations selectively modified by ADRY-agents or trypsin treatment or special dark-adaptation regimes led to the conclusion that the difference spectra of the $S_i \rightarrow S_{i+1}$ ($i = 0, 1, 2$) do not markedly differ as will be outlined elsewhere (Weiss and Renger, in preparation).

Further experiments are required for the spectral characterization of all redox transitions in the water oxidizing enzyme system and the consistent interpretation of the data in order to achieve an unambiguous identification of the S_i states.

VII. The apoenzyme of the water oxidizing enzyme system

The water oxidizing enzyme complex is located within the membrane and exposed to the thylakoid lumen. This arrangement had hindered the discovery of polypeptides involved (directly or indirectly) in water oxidation. Significant progress was achieved only after the development of mild procedures that allowed the isolation of thylakoids with inverted membrane polarity

[ISO-thylakoids, ref. 105] and of PS II particles which remained highly active in oxygen evolution [106, also see 106a]. Treatment of these preparations with NaCl at high concentrations (~ 1 M) caused the release of two polypeptides with 17 kDa and 23 kDa molecular weight and a concomitant decrease of oxygen evolution without any loss of bound manganese [for reviews, see refs. 107, 108]. Rebinding of the 23 kDa polypeptide partially restored the activity for water oxidiation, whereas the 17 kDa unit was found to be without any effect [but see ref. 109]. The role of these polypeptides and of 33–34 kDa polypeptides will be discussed fully in a forthcoming review by C. F. Yocum in *Photosynthesis Research.*

Based upon single turnover flash experiments and the lack of any functional group or metal center, it was concluded that the 23 kDa polypeptide supports the functional connection of the water oxidizing enzyme system with RC II via structural effects that could poise the redox potential of Z [107, 110, 111]. Latest data on the correlation between the concentration dependence of the Cl^--effect (vide supra) and the content of the 23 kDa polypeptide in ISO-thylakoids led to the conclusion that the 23 kDa unit markedly enhances the affinity of the native Cl^--binding site in the water oxidizing enzyme system [112]. A third polypeptide having a molecular weight of 33 kDa was lost by Tris-washing or urea treatment [108, 113]. This was accompanied by a loss of manganese from the preparations. Based upon the stoichiometric relation between bound manganese and membrane bound 33 kDa polypeptide, it was inferred that this polypeptide contains the functional (binuclear?) manganese group for water oxidation [108].

However, latest findings of $CaCl_2$-induced release of the 33 kDa polypeptide without the loss of manganese [114] question the above-mentioned assignment. Partial restoration of oxygen evolution after rebinding of the 33 kDa polypeptide [115] might suggest a regulatory rather than a direct functional role of this unit. On the other hand, based on studies of several mutant phenotypes of a green alga *Scenedesmus obliquus,* the functional manganese was inferred to be incorporated into a 34 kDa polypeptide [for a review see ref. 116], which has caused some 'confusion' regarding the role of the above-mentioned 33 kDa polypeptide. However, O_2-evolving PS II maize preparations were shown to contain an intrinsic membrane protein of 34 kDa as well as a surface attached 33 kDa unit [117]. Manganese was inferred to interact with both these polypeptides [117]. It appears that Mn may be tightly associated with a 32–34 kDa polypeptide if extracted in oxidizing conditions [Abramowicz et al., 117a]. An analysis of the effect of $CaCl_2$ on manganese release of PS II particles led to the conclusion that the 33 kDa polypeptide sustains a conformational state around the native manganese binding site that is required for its stabilization. It was suggested that Ca^{2+} as well as the 33 kDa polypeptide maintain the conformation around the Mn-binding sites required for O_2 evolution [118].

Trypsination of ISO thylakoids revealed a strong pH-dependence of the

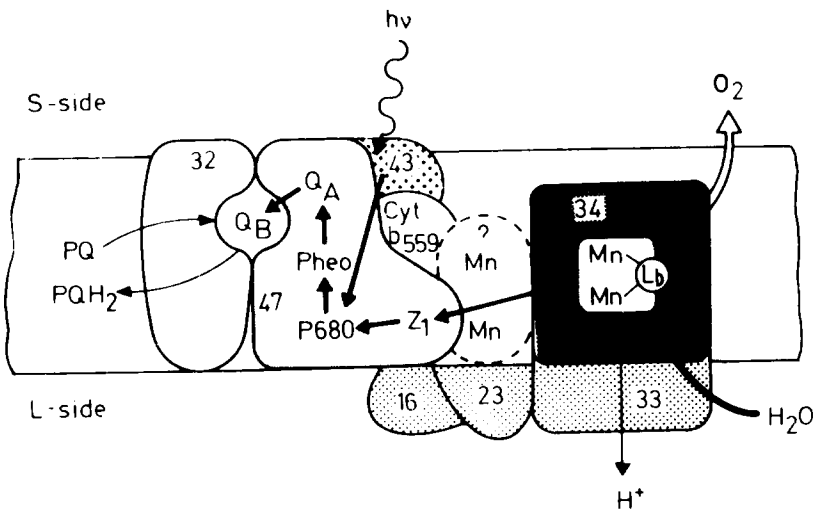


Figure 4. A simplified scheme of the functional and structural organization of photosynthetic water oxidation. Numbers indicate the molecular weight of the polypeptides (kDa); Q_A, Q_B = primary and secondary plastoquinone acceptor; Cyt-b_{559} = cytochrome b_{559}; other components are explained in the text.

degradation of the oxygen evolving capacity [119] as well as of manganese release (Völker M., Ono T., Inoue Y. and Renger G., in pre.) indicating pH-dependent conformational changes that modify the susceptibility to trypsin.

Summarizing the above-mentioned data, the model depicted in Figure 4 [see also ref. 119] has been derived with the assumption that an intrinsic 34 kDa polypeptide is the apoenzyme of the functional binuclear manganese center, but the 23 kDa and 33 kDa units are indispensible for the functional connection with RC II. This connection is likely to be regulated in a specific manner by divalent cations. First evidence for a functional role of Ca^{2+} was found in cyanobacteria [120; also see 120a]. Sophisticated work on photoactivation revealed a binding site for Ca^{2+} [121]. Furthermore, Ca^{2+} was found to cause partial reactivation of oxygen evolution in PS II particles depleted of their 17 kDa and 23 kDa polypeptides by salt treatment [114, 122] and in EGTA treated ISO thylakoids [123]. Data of Miyao and Murata [123a] also suggest that Ca^{2+} can substitute for the 23 kDa polypeptide. Also, Nakatani [123b] has shown that in the presence of high concentrations of Cl^- and Ca^{2+}, 17 kDa and 23 kDa polypeptides are not required for O_2 evolution. Ono and Inoue [123c] showed a parallel in rebinding of 33 kDa polypeptide with restoration of O_2 evolution in their system. However, the exact role of Ca^{2+}, which can be replaced in some cases by Mg^{2+} or Mn^{2+} but not in others [117], remains to be examined in future studies. Furthermore, as chloride plays an essential role in the oxygen-evolving

capacity and at least the 23 kDa subunit was found to affect the chloride affinity [112], it remains to be clarified to what extent the nature of the anion influences the above-mentioned salt effects. Another very interesting phenomenon is the discovery [60] that after extensive dark adaptation the water oxidizing enzyme system attains a resting conformation, where the manganese site is insensitive to different treatments that block O_2-evolution. Only in the active conformation, persisting after dark adaptation in the minute range after illumination, the Mn site is open to interaction with H_2O, amines and chloride. The underlying mechanism of this effect is unknown.

Taking into account all the data known thus far, it appears that the water oxidizing enzyme system consists of three different entrinsic subunits (17 kDa, 23 kDa, 33 kDa) and an intrinsic 34 kDa polypeptide being very likely the site of the functional (binuclear?) manganese center. In this regard, it is very interesting to compare the water oxidizing enzyme system with cytochrome oxidase, which catalyses the reverse reaction (see equations (1)). Cytochrome oxidase of higher organisms consists of at least 7 different subunits [for a review see 2], but the catalytic centers Fe(Cyt-a_3) and Cu_B are associated only with subunit I, which is in contact with subunit II [124]. Furthermore, it was shown that at early stages of evolution the catalytic subunits I and II were 'invented', and remained highly conserved [124], whereas at later stages additional subunits were developed that obviously function as regulatory entities [125]. Interestingly enough, also for the reverse process, i.e. water oxidation, the basic principles of the mechanism must have remained unchanged since the early discovery of its realization in the ancient cyanobacteria [126]. Therefore, it would be worthwhile to analyze whether the subunit structure of the water oxidizing enzyme system has been changed during the evolutionary development by additional regulatory units as in cytochrome oxidase. With respect to the size it is interesting to note that the catalytic subunit I of cytochrome oxidase in many organisms has a molecular weight of 32–40 kDa [124] thereby resembling the catalytic subunit of the water oxidizing enzyme system. Furthermore, the catalytic subunit I is an intrinsic membrane protein, whereas most of the regulatory subunits are surface exposed. This reveals striking similarities in the overall organization scheme of cytochrome oxidase and the water oxidizing enzyme system.

A comparison of the water oxidizing enzyme system with oxidases (laccase, cytochrome oxidase) poses a further interesting problem. In both enzyme complexes the catalytic binuclear center is functionally connnected with other redox groups, containing two metal centers, that mediate the electronic coupling with the external donor components [for a review, see ref. 40]. Accordingly, a similar situation could be anticipated also for the water oxidizing enzyme system. In this regard, it would be very attractive to speculate about the existence of a further redox group that mediates the connection with RC II via Z. A likely candidate could be another man-

ganese group which would provide an explanation for the heterogeneity of the manganese pool, as is schematically depicted in Figure 4.

VIII. Concluding remarks

Based upon our present knowledge, photosynthetic water oxidation occurs at a multienzyme complex that contains a key functional group – a special chlorophyll a (P680) – for the generation of sufficiently oxidizing redox equivalents (the redox potential of $P680^+/P680$ has been estimated to be 1.12 eV, [127]) and a binuclear (?) manganese center that catalyzes the redox sequence leading from water to molecular oxygen. It is highly likely that four manganese atoms are essential for water oxidation, with two involved in the catalytic site, and the other two in the oxidation of this site. The P680 and the catalytic manganese centers are incorporated into protein matrices and are coupled with other redox centers. Although the general function and organization of the O_2-evolving system is now available, yet the many details of the molecular mechanism and its regulatory control are still missing. It is of special interest to note that for cytochrome *b* 559, which is associated with the O_2-evolving system, we have not yet found a clearly defined role at physiological temperatures. (See Widger et al. [128] for the physico-chemical nature of this cytochrome, and Butler and Matsuda [129] for a possible hypothesis for its function.)

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