Review

Metallo-radical hypothesis for photoassembly of (Mn)₄-cluster of photosynthetic oxygen evolving complex¹

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Abstract

A new hypothetical mechanism is proposed for photoassembly of the (Mn)₄-cluster of the photosynthetic oxygen evolving complex (OEC). In this process, a neutral radical of Y Z tyrosine plays a role in oxidizing Mn²⁺ associated with an apo-OEC, and also in abstracting a proton from a water molecule bound to the Mn²⁺ ion, together with D1-His190. This is in a similar fashion to the metallo-radical mechanism proposed for photosynthetic water oxidation by the (Mn)₄-cluster. The model insists that a common mechanism participates in the photoassembly of the (Mn)₄-cluster and the photosynthetic water oxidation. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

In photosynthesis, absorption of a photon induces the excited state of the photosynthetic reaction center followed by charge separation, and the separated electron produces the reducing power required for the formation of NADPH as well as ATP. For successive charge separation by photons to occur, the oxidized reaction center must be re-reduced by an electron which may derive from various electron sources. Water is used as an electron source in photosystem (PS) II, and O₂ is released as a consequence of four-electron oxidation of two water molecules. Oxidation of water is carried out by a photosynthetic oxygen evolving complex (OEC) composed of inorganic cofactors including Mn, Ca and Cl ions, and redox-active tyrosine of the D1 protein (Y Z tyrosine), which are thought to be closely associated in terms of both structure and function, with a stoichiometry of 4:1:2:1 per unit of the OEC. Four Mn ions constitute a tetranuclear cluster ((Mn)₄-cluster) that functions as a catalytic center for water oxidation by accumulating the oxidizing equivalents produced by successive absorption of four photons (for reviews, see [1–4]). The (Mn)₄-cluster is oxidized by one equivalent upon absorption of each photon by the PS II reaction center in response to the advancement of four stable intermediates Sᵢ (ᵢ = 0–3) which

Abbreviations: Chl, chlorophyll; DCIP, 2,6-dichlorophenolindophenol; DPC, diphenylcarbazide; OEC, oxygen evolving complex; PS, photosystem; QA, primary quinone acceptor of photosystem II; YZ, redox-active tyrosine of the D1 protein; YZox, oxidized Y Z; YZ*, neutral radical of Y Z

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¹ This paper is dedicated to Prof. M.P. Klein of blessed memory.

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are kinetically isolated. The (Mn)₄-cluster lays in the lowest and highest oxidation states in S₀ and S₄, and is reduced upon the S₃(S₄)S₀ transition with the concurrent release of O₂ [2,5–7]. The OEC is located at the luminal side of the PS II protein complex but the detailed structure of the OEC and the reaction mechanism of water oxidation remain largely unknown.

Light is required not only for the energy source for photosynthetic reactions but also for regulating the photosynthesis process in numerous respects. However, light is not in principle required for the biogenesis of the machinery functioning for photosynthesis. In fact, chloroplasts can be normally developed under complete darkness in algae and gymnosperms. These dark-grown plants, however, lack the capability of photosynthetic O₂ evolution [8,9]. Quantification of the abundance of Mn showed that the chloroplasts do not retain a stable Mn pool [8], indicating that the assembly of the (Mn)₄-cluster is impaired under darkness. However, O₂ evolution activity is induced by illuminating the plants with PS II light concomitant with the incorporation of four Mn ions [8,9]. This light-dependent assembly process of the (Mn)₄-cluster is called ‘photoactivation’. In angiosperms, chloroplasts do not develop under dark conditions due to the presence of light-dependent signal transduction paths regulating the chloroplasts development. Development proceeds when the chlorophyll (Chl) biosynthesis is supported in the plants being illuminated with intermittent light [10,11]. The chloroplasts, thus formed, cannot evolve O₂ under certain intermittent light conditions and the capacity for O₂ evolution is only developed by illumination [10,12,13]. Light is also required for the development of O₂ evolution in algal cultures grown under Mn-deficient conditions after the supplementation of Mn⁴⁺ [14–16]. Therefore photoactivation is the only process for generating the (Mn)₄-cluster and the universal process among organisms performing oxygenic photosynthesis. By the same process, the (Mn)₄-cluster can be re-assembled in samples in which the (Mn)₄-cluster has been extracted by treatment with Tris [17], NH₂OH [18–20], or ascorbate plus lipophilic chelator [21]. Supplementation of Mn²⁺, Ca²⁺, Cl⁻ and artificial electron acceptors is required to obtain maximum reactivation yield [22].

Photoactivation (photoassembly of the (Mn)₄-cluster) has been demonstrated in chloroplasts [13,17], PS II-enriched membranes [19,20], PS II core complexes [23] and PS II subcomplexes devoid of CP43 [24]. Therefore, the photoassembly of the (Mn)₄-cluster needs no special factors or reactions except for those of PS II itself, and must be an intrinsic and inherent attribute of PS II.

The mechanism for photoactivation is not clearly defined at this point in time largely due to the lack of knowledge about the structure of the (Mn)₄-cluster. In other words, photoactivation may help in understanding the function and structure of the OEC. In the present paper, we briefly review some aspects of photoactivation, and propose a new model for the photoassembly of the (Mn)₄-cluster based on a metallo-radical process involving Y₂ tyrosine.

2. Kinetic analyses and derived model of photoactivation

Models of the process (mechanism) of photoactivation have been proposed on the basis of kinetic analyses of the appearance of O₂ evolution capability during illumination. Since there is usually a linear relationship between O₂ evolution activity observed and the relative proportion of OECs acquiring the active (Mn)₄-cluster, we can follow the course of the assembly of the (Mn)₄-cluster by monitoring O₂ evolution activity during the course of photoactivation. The following fundamental kinetic results have been obtained by Cheniae and Martin [16] by analyzing the photoactivation process in Mn-deficiently grown cyanobacteria (Anacystis): (1) the rate of photoactivation is proportional to the number of inactive OECs and is a first-order process. (2) The dependence curve of the rate of photoactivation on light intensity (rate of quantum absorption) shows a lag in very weak light, and saturates at one-tenth of the intensity needed for photosynthetic saturation. (3) The quantum efficiency of photoactivation is very low, but constant and optimal within a narrow range of light intensities, and markedly declines at both lower and higher intensities. These results imply that the photoactivation is a multi-quantum process involving unstable intermediate states and one or more slow rate-limiting dark steps. These processes have been further studied by using flashing light with
various periods of darkness time between flashes; the yield of photoactivation increased with increase in the dark interval until a maximum yield was reached, and then decreased with further increase of the interval [16]. Similar results have been obtained in all of the photoactivation systems examined so far [8,12,18,25–27], showing that the basic reaction mechanism is identical.

Based on these results, it has been proposed that photoactivation proceeds via at least two photochemical reactions with a rate-limiting dark reaction between them [28] (see Fig. 1). The inactive apo-OEC (State A) is converted by the first photochemical reaction to the first intermediate state (State B), which is not sensitive to light. This intermediate is transformed into another intermediate (State C) by the rate-limiting dark reaction. The second intermediate ends up as an active OEC by the second photochemical reaction, although the second intermediate is unstable and decays to State A unless the second photochemical reaction has not taken place. Mn$^{2+}$ bound to the apo-OEC is assumed to be oxidized to Mn$^{3+}$ by the photochemical reactions.

The flash-interval dependence curve is the result of the cumulative effect of multiple flashes due to the low quantum efficiency of photoactivation. In order to confirm that the two successive photochemical processes are sufficient for photoactivation, experiments have been performed using repetitive clustered flashes with a long interval between the clusters. The clusters contain a varying number of flashes [8,16,26,27]. Appreciable O$_2$ evolution is developed only when the cluster contains more than two flashes [8,16,26,27], and the photoactivation yield from clusters that consist of $\geq$ 3 flashes is comparable to that from clusters containing two flashes [27]. These results strongly indicate that only two photochemical processes occur in photoactivation. This view is confirmed directly by the observation that O$_2$ activity is not restored by one flash but restored appreciably by $\geq$ 2 flashes [29]. The presence of reducing agents is inhibitory for photoactivation [8,27,30], owing mainly to the stimulated deactivation of the intermediates [27]. Therefore, reduction of Mn$^{3+}$ to Mn$^{2+}$ is assumed to be responsible for the dark decay from State C to State A as well as from State B to State A. Studies of the respective steps with more detailed kinetic resolution have been allowed by the development of an in vitro photoactivation system by the use of PS II-enriched membranes [19,20] and introduction of an O$_2$ electrode with high sensitivity and fast response time [21,31–33].

Fig. 1 shows a kinetic model of photoactivation mainly based on [19]. Photoactivation is composed of the following reaction steps: [step 1] Binding of Mn$^{2+}$ to a high-affinity binding site for Mn$^{2+}$ in the apo-OEC (State A). [step 2] Photo-induced oxidation of the bound Mn$^{2+}$, forming an unstable apo-OEC-Mn$^{3+}$ intermediate (State B). [step 3] Binding of another Mn$^{2+}$ after a rate-limiting conformational rearrangement of the apo-OEC induced by the first photochemical event, forming the second intermediate (State C). [step 4] Oxidation of the second Mn$^{2+}$ by the second photoreaction, followed by the formation of a Mn dimer. [step 5] Binding and ligation of other two Mn$^{2+}$ to complete the (Mn)$_4$-cluster. This
step must include several light and/or dark reaction processes although details of these reactions were not defined yet. Ca\(^{2+}\) may participate in the change in protein conformation from State B to State C [32]. Proton release from the first intermediate is suggested by the effect of buffer capacity on the yield of photoactivation and the large decrease in the halftime of photoactivation with increasing buffer pH [31].

3. Mn\(^{2+}\) ion binding to the apo-OEC and its photooxidation

The Mn\(^{2+}\) ion but not the Mn-EDTA complex can donate an electron to PS II in various types of Mn-depleted PS II preparations. Supplementation of Mn\(^{2+}\) accelerates the decay course of the oxidized Y\(_{Z} \) (Y\(_{Z}^{ox}\)) EPR signal and/or suppresses the signal intensity under multiple flash [34,35] or single flash [36] conditions, indicating that Mn\(^{2+}\) is directly oxidized by Y\(_{Z}^{ox}\). The presence of several binding sites for Mn\(^{2+}\) with different affinities in the apo-PS II was suggested by kinetic studies of Mn\(^{2+}\) oxidation monitored by 2,6-dichlorophenolindophenol (DCIP) reduction, Chl fluorescence and Mn\(^{2+}\)/diphenylcarbazide (DPC) assay [37–43]. It has not, however, been determined whether these reflect heterogeneity of a single site or the presence of different sites with different binding affinities. Among these sites, a high-affinity site for Mn\(^{2+}\) (K\(_{m}\) \(\approx\) several \(\mu\)M) is prevalently observed by monitoring the decay of flash-induced Chl fluorescence [36,42] and the Y\(_{Z}^{ox}\) signal [36], and by Mn\(^{2+}\)/DPC [39,41,43] and DCIP reduction [37,38] assays. The supplementation of reductant is required for the detection of this site by DCIP reduction assay under continuous light [37,38]. This is presumably due to the replacement of Mn\(^{3+}\) formed at the binding site by an external Mn\(^{2+}\) becoming rate-limiting in the reaction. The observed affinity for Mn\(^{2+}\) oxidation is comparable to the apparent K\(_{m}\) (\(\approx 4\) \(\mu\)M) of Mn\(^{2+}\) for photoactivation [40]. It is inferred that the quantum yield for Mn\(^{2+}\) oxidation at this high-affinity site is high since primary quinone acceptor of PS II (Q\(_{A}\)) Y\(_{Z}^{ox}\) recombination after flash excitation is completely suppressed by Mn\(^{2+}\) [36,42]. Illumination of the Mn-depleted PS II by weak light specifically inhibits the formation of a Y\(_{Z}^{ox}\) EPR signal concurrent with the inhibition of Mn\(^{2+}\) oxidation at the high-affinity site. It also inhibits the capability for photoactivation although neither electron donation from DPC nor from Mn\(^{2+}\) at higher concentrations is affected [40,44–47]. Supplementation of \(\approx 1\) Mn\(^{2+}\)/OEC suppresses the photoinhibition [46]. All these results are compatible with the view that Y\(_{Z}^{ox}\) is responsible for the oxidation of the bound Mn\(^{2+}\) and that the photo-oxidation of Mn\(^{2+}\) at the high-affinity site can be regarded as the first photochemical process of photoactivation.

Analyses of Mn\(^{2+}\) oxidation under continuous light [48] or Y\(_{Z}^{ox}\) EPR signal formation under multiple flashes [49] indicated that Y\(_{Z}^{ox}\) can be reduced efficiently by electron donation from Mn\(^{2+}\) at a concentration equivalent to two Mn\(^{2+}\)/PS II. Mn\(^{2+}\)/DPC assay suggested the presence of four high-affinity sites for Mn\(^{2+}\) in the apo-OEC although one of the sites binds a photo-oxidizable Mn\(^{2+}\) specifically [41,50], and half of the original sites are lost in the LF-1 mutant of Scenedesmus obliquus [50,51]. An EPR signal probably arising from a spin-coupled binuclear Mn(II,II) has been observed in the dark by the addition of Mn\(^{2+}\) to the apo-OEC at a concentration equivalent to two Mn\(^{2+}\)/PS II in the presence of Ca\(^{2+}\) [49]. It has been proposed that Ca\(^{2+}\) organizes the Mn(II,II) dimer by interacting directly via solvent or protein-derived bridging ligands and that the Ca\(^{2+}\)-induced Mn(II,II) dimer serves as a precursor for the functional (Mn\(_{4}\))-cluster during photoactivation [49]. This may support the view that some type of multi-cluster will be formed upon binding of Mn\(^{2+}\) ions before illumination necessary for photoactivation. On the other hand, direct quantification of the amount of Mn\(^{2+}\) bound to the apo-PS II in the dark under conditions equivalent to those for photoactivation inferred that the apo-OEC binds only one Mn\(^{2+}\)/PS II. This is sufficient for the complete reduction of Y\(_{Z}^{ox}\) induced by flash excitation [36]. Furthermore, photoactivation occurs efficiently when mononuclear Mn complexes with Niten and Salhxn ligands are used instead of Mn\(^{2+}\) [52]. These observations favor the idea that only one Mn\(^{2+}\) ion is associated with the apo-OEC at the first photochemical step in the photoactivation process. Other three Mn\(^{2+}\) ions cannot associate with the apo-OEC due to the absence of other Mn\(^{2+}\) sites or low affinity
of the sites for Mn$^{2+}$. Therefore, the various types of Mn$^{2+}$ binding observed by kinetic analysis may not directly participate in the process of photoactivation. Since four manganese ions are necessary for the formation of the native (Mn)$_4$-cluster, high-affinity sites for Mn$^{2+}$ or Mn$^{3+}$ should be newly created as a consequence of the oxidation of the first Mn$^{2+}$ ion. It is inferred that this process corresponds to the proposed dark rate-limiting step between the two photochemical reactions in the kinetic model of photoactivation shown in Fig. 1 [36].

Chemicals that modify a carboxyl residue inhibit the Mn$^{2+}$ oxidation at the high-affinity site [53]. They also abolish some of the high-affinity sites detected by Mn$^{2+}$/DPC assay [41,50,51,53,54] as well as the capability of photoactivation [55], but the capability of photooxidation of DPC is well preserved [41,50,51,53-55]. This indicates that acidic amino acids are involved in the high-affinity Mn$^{2+}$ binding process and/or the ligation of the (Mn)$_4$-cluster although the chemicals may affect photoactivation via an effect on Ca$^{2+}$ binding. Analyses of the site-directed mutants at aspartate 170 of the D1 protein (D1-Asp170) reveal that this residue participates in the binding and oxidation of exogenous Mn$^{2+}$ [42,56], and the stability or assembly of the (Mn)$_4$-cluster [56]. Both the processing mutant and truncation mutant of the C-terminus of the D1 protein in Synechocystis 6803 [57] as well as the non-processing mutant of $S$. obliquus (LF-1 mutant) [58,59] lose the ability to assemble the (Mn)$_4$-cluster [58-61], but can oxidize exogenous Mn$^{2+}$ normally [57]. Therefore, D1-Asp170 is responsible for the binding and oxidation of Mn$^{2+}$ at the unique high-affinity site existing in the apo-OEC in the first photochemical process of photoactivation. The free carboxyl-terminus of the D1 protein participates in the ligation of the (Mn)$_4$-cluster but does not contribute to the binding and oxidation of this Mn$^{2+}$. The carboxyl-terminus may become accessible to aqueous Mn$^{2+}$ only after the change in protein conformations induced by the Mn$^{2+}$ oxidation in the first photochemical process of photoactivation. However, it can be accessed by the D1 processing enzyme in a solution before being processed.

Oxidation of exogenous Mn$^{2+}$ by PS II is inhibited by the presence of high concentrations of cations, such as Ca$^{2+}$, Mg$^{2+}$ [34,36] and K$^+$ [34]. It was shown that the inhibition depends on cationic strength, indicating that the inhibition by these cations can be ascribed to the electrostatic effect which reduces the local concentration of Mn$^{2+}$ around its binding site and increases the apparent $K_m$ value, although Na$^+$ did not follow the dependence [34]. Ca$^{2+}$ and Mg$^{2+}$ equally effected an increase in the concentration of Mn$^{2+}$ required for binding to the unique high-affinity site in the apo-OEC [36]. On the other hand, kinetic analysis of the inhibitory effects of alkali metal cations and divalent cations on the course of photoactivation suggested cation-specific binding to the high-affinity Mn$^{2+}$ site of the apo-OEC [33]. The apparent inconsistency among the literature may be partly attributable to the occupation of the Ca$^{2+}$-binding site by another cation which inhibits photoactivation. Alternatively, this may be interpreted to mean that a Mn$^{2+}$ ion is not an active species for reducing Y$\alpha$ $\beta$ [33].

It has been inferred that alkali metal cations with larger ionic radii are bound to the high-affinity site for Mn$^{2+}$ with increasing affinity, while divalent cations with larger ionic radii are bound to the site with decreasing affinities [33]. Binding affinities for the divalent cations (Mn$^{2+}$, Ca$^{2+}$ and Sr$^{2+}$) correlate with the hydrolysis constant for formation of the metal hydroxide by hydrolysis of water. This suggests that Mn$^{2+}$ is bound to the high-affinity site as [Mn(II)-OH]$^+$ which is oxidized in photoactivation [33]. Cs$^+$ binds to the high-affinity Mn$^{2+}$ binding site with a slightly higher affinity than Mn$^{2+}$, and inhibits Mn$^{2+}$ oxidation under continuous illumination [33]. It has been inferred that the binding site for Mn$^{2+}$ is designed for Mn$^{2+}$ as well as [Mn(II)-OH]$^+$, whose size and charge are comparable with those of Cs$^+$. Furthermore, this species could be a precursor for the o xo-bridges between Mn(III) and Mn(IV) in the native (Mn)$_4$-cluster in the OEC [33].

4. Metallo-radical mechanism for photoassembly of the (Mn)$_4$-cluster

The involvement of the formation of [Mn(II)-OH]$^+$ species in the process of photoactivation is an attractive concept. It is particularly worth considering since no proposed model for photoactivation had explained why the di-μ-oxo-bridged Mn dimer
structure proposed for the native Mn-cluster [2,62] can be formed as a consequence of Mn\(^{2+}\) oxidation. The [Mn(II)-OH]\(^+\) formation may be compatible with the experimental observation that the apparent affinity of Mn\(^{2+}\) to the apo-OEC determined by the rate of Mn\(^{2+}\) oxidation increases with increasing pH [35,36] and the photoactivation half-time decreases with increasing pH [31]. However, the formation of [Mn(II)-OH]\(^+\) from [Mn(II)-OH]_2\(^{2+}\) by hydrolysis of bound water is very unlikely in aqueous solution at neutral pH values where the photoactivation yield is maximum. This difficulty could be overcome by assuming either the presence of a base that can serve as an acceptor of a proton from the bound water molecule or markedly high affinity of [Mn(II)-OH]\(^+\) to the site as pointed out in [33]. The latter possibility seems to be less likely since apparent affinity of the site for Mn(II) (Mn\(^{2+}\) or [MnOH]\(^+\)) estimated by Mn\(^{2+}\) oxidation has been reported to be in the \(\sim\)\(\mu\)M range [36-38,42] which may be insufficient for promoting the formation of the [Mn(II)-OH]\(^+\) species. This may lead us to consider the first possibility and we should allow for what type of base could be available on the donor side of Mn-depleted PS II. The base must be strong enough for accepting H\(^+\) generated by a water hydrolysis reaction with \(pK_a\) of 10.5.

Metallo-radical mechanism models of the photosynthetic O\(_2\) evolution propose the direct involvement of YZ in the water oxidation which abstracts a H-atom from water molecules in each of the S state transitions [63-65]. In these models, YZ functions as a photogenerated base: oxidation of YZ by P_{680} triggers the phenolic proton transfer to a proximal base due to the markedly low \(pK_a\) (\(\sim\)2) of the cation radical of tyrosine [66], generating a neutral tyrosyl radical, YZ". Subsequent electron transfer from the (Mn)\(_4\)-cluster to YZ" results in a one step advance of the (Mn)\(_4\)-cluster to the S\(_{e+1}\) state and a deprotonated YZ abstracts a proton from water or hydroxide bound to the (Mn)\(_4\)-cluster [63]. Alternatively, YZ" is reduced by a single H-atom transfer [64,65]. On the basis of modeling studies that show the presence of D1-His190 in the close proximity of YZ [67-69] as well as site-directed mutagenesis studies [70-75], D1-His190 is believed to serve as the proximal base. The basic properties of YZ that lead to the metallo-radical mechanism for photosynthetic O\(_2\) evolution are:

- (i) oxidation of YZ as a neutral radical [77-79], (ii) a high degree of disorder in the dihedral angle of C-H bond of \(\beta\) methylene with respect to tyrosine phenoxyl plane in YZ\(_{ox}\) [63,65,80]. The former indicates that YZ\(_{ox}\) is deprotonated during oxidation, and the latter suggests a high degree of flexibility of the phenoxyl sidechain and is compatible with the view that YZ participates in the coupled electron/proton transfer from the (Mn)\(_4\)-cluster. The proposed function of YZ in the metallo-radical mechanism is pertinent if it is the base for promoting the [Mn(III)-OH]\(^{2+}\) formation.

We note that the metallo-radical mechanism has never been confirmed directly by experimental evidence. In this context, it has been reported that a proton is released from peripheral amino acids but not from YZ\(_{ox}\) in oxygen evolving PS II, and inferred that YZ plays a role in water oxidation process mainly as an electrostatic promoter [81-83]. However, the properties and reactions of YZ supporting the metallo-radical mechanism could be applicable to photoactivation with minor revision since these have derived from the studies in Mn-depleted PS II. In the inactive Mn-depleted PS II, the rate of proton release into the bulk water is coincident with that of YZ to P_{680} electron transfer which reveals a large H/D isotope effect [82]. Furthermore, little local electromorphism does not accompany YZ oxidation [82]. These indicate that the electron transfer is coupled with proton transfer upon YZ oxidation in the PS II retaining no (Mn)\(_4\)-cluster.

Here, we propose a new mechanistic model of photoassembly of the (Mn)\(_4\)-cluster, in which YZ" acts as a hydrogen atom abstractor that facilitates [Mn(III)-OH]\(^{2+}\) formation by promoting the deprotonation of a water molecule bound to Mn(II) at the unique site (Fig. 2).

4.1. Step 1

The Mn(II) aqua dication ([Mn(II)-OH]_2\(^{2+}\)) is bound to the unique high-affinity site which exists in the close proximity of YZ before light illumination. This results in the initial state that is corresponding to ‘State A’ in the kinetic model shown in Fig. 1.

In this state, YZ is protonated due to its estimated \(pK_a\) of \(\sim\)10.3 in Mn-depleted PS II [76]. In this
model, the high-affinity binding site for Mn$^{2+}$ oxidation exists in the close proximity of Y$_Z$. Although the location of the high-affinity site in the apo-OEC has not been identified, this is not an extravagant idea as the site is believed to overlap partly with the native ligation site of the (Mn)$_4$-cluster, which is estimated to be 3.5–4.5 Å [63,84], 7.7–11.5 Å [85–88] or 15–20 Å [89] away from Y$_Z$. Furthermore, site-directed mutagenesis studies suggest the involvement of D1-Asp170 in ligation and/or oxidation of Mn$^{2+}$ at the high-affinity site [42,56]. From a computer modeling study, the distance between Y$_Z$ and D1-Asp170 is estimated to be 6.3 Å [68], which is compatible with the length of the hydrogen bond (2.6–2.8 Å) existing between a Mn$^{2+}$-bound water molecule and Y$_Z$ if D1-Asp170 composes the binding site for Mn$^{2+}$ or exists in very close vicinity of the Mn$^{2+}$ binding site. Several lines of evidence indicate that Y$_Z$ (Y$_{Ox}^Z$) is in rapid communication with the solvent water and the accessibility is enhanced by the depletion of the (Mn)$_4$-cluster [90–92].

4.2. Step 2

After the first illumination, Y$_Z$ is oxidized by P$_{680}^+$ concurrently with the transfer of a phenolic proton...
to D1-His190, yielding a neutral tyrosyl radical, Y$_Z^\bullet$.

Based on the close similarity in the EPR line shapes of Y$_Z^\alpha$ and Y$_D^\alpha$ as well as a comparison with model tyrosines, Y$_D^\bullet$ formed in Mn-depleted PS II is a neutral radical [1] although the spin density distribution may be more different than for a typical neutral radical [93]. Deprotonation of Y$_{ox}$ into the bulk water has been demonstrated in Mn-depleted inactive OEC, and the process is controlled by a residue with a $pK_a$ value of 7 [82]. D1-His190 is reported to function as a proton acceptor for Y$_Z$ in Mn-depleted PS II although the interaction between Y$_Z$ and D1-His190 is altered to be less coupled compared with interaction in the presence of the (Mn)$_4$-cluster [75,76]. Many of the D1-His190 mutants are unable to evolve oxygen presumably due to the impairment of the assembly of the (Mn)$_4$-cluster [71,72]. This must be attributed to impaired Mn$^{2+}$ oxidation capability at the high-affinity site in the mutants [94] since Y$_Z$ oxidation by $P_{680}^\bullet$ is largely suppressed when the absence of D1-His190 keeps Y$_Z$ protonated [74–76].

4.3. Step 3 and step 3’

The neutral Y$_Z$ radical (Y$_Z^\bullet$) is re-reduced by an electron donation from the bound Mn(II) and proton donation from a water molecule bound to Mn(II), leaving [Mn(III)-OH]$^{2+}$ and a protonated Y$_Z$. This results in the first intermediate state that is corresponding to ‘State B’ in the kinetic model shown in Fig. 1.

The implication of Y$_Z^\bullet$ as the oxidant of Mn$^{2+}$ has been indicated by the stimulation of Y$_Z$ reduction [34–36] and the prevention of Q$_A$ Y$_Z$ recombination by Mn$^{3+}$ [36,40,42,43,54]. Y$_Z^\bullet$ is reduced in a partially first-order manner with a half-time of 300–500 μs at pH 7.5 [34], which is comparable to the reduction rate by the (Mn)$_4$-cluster in an intact OEC in the S$_0$, S$_1$ and S$_2$ states [1]. Above the Mn$^{2+}$ concentration that leads to one Mn$^{2+}$ ion binding to the apo-OEC, Y$_Z$ reduction by Mn$^{2+}$ does not follow a second-order process. This indicates that Y$_Z^\bullet$ is directly reduced by Mn$^{2+}$ bound to the unique site [36]. Consistent with this, Y$_Z$ is selectively impaired by illuminating the apo-OEC in the absence of Mn$^{2+}$ concomitant with the loss of the capability for both Mn$^{2+}$ oxidation at the high-affinity site and photoactivation [40,44,45].

The process of Y$_Z^\bullet$ reduction must be coupled with the reprotonation of Y$_Z$ because Y$_Z$ is a strong base. In Mn-depleted PS II, Y$_Z^\bullet$ is reduced by an electron donation from Q$_A$ in the absence of Mn$^{2+}$. The Y$_Z^\bullet$ Q$_A$ recombination is inhibited in D1-His190 mutants, indicating that the process requires proton donation to Y$_Z^\bullet$ from D1-His190 or another base via D1-His190 [74–76]. Upon the reduction of Y$_Z^\bullet$ by the bound Mn(II) aqua dication ([Mn(II)-OH]$^{2+}$), a proton is donated from Mn(II)-coordinated water that is acidified by ligating Mn(II), leaving [Mn(III)-OH]$^{2+}$ at the binding site. In this process, no change in net charge occurs during the Mn(II) oxidation. This reaction is thermodynamically feasible since the bond dissociation energy for water (113.4 kcal/mol) lowers by ligating to Mn at a value of 78.0–80.9 kcal/mol. This is comparable to the value for the tyrosyl O–H bond (81.5 kcal/mol) as discussed in detail for the metallo-radical mechanism of O$_2$ evolution [95–97]. The protonated Y$_Z$ prevents [Mn(III)-OH]$^{2+}$ from reprotonation and stabilizes it. The coupled transfer of an electron and a proton may be responsible for a high quantum yield for the Mn$^{2+}$ oxidation.

A disordered hydrogen bonding or the hydrogen-bonded chain via water molecule(s) between Y$_Z$ and D1-His190 in Mn-depleted PS II [75,76] makes proton abstraction from the coordinated water a more likely process, compared with abstraction from D1-His190. This view is consistent with the finding that the rate of electron donation from the bound Mn(II) is much faster than that from Q$_A$ [34–36,40–43,54]. The disordered hydrogen bond network allows considerable flexibility in the Y$_Z$ tyrosine sidechain that facilitates coupled transfer of the electron and proton. The regeneration of the protonated Y$_Z$ may be coupled with the proton transfer from D1-His190 to lumen. The rate of Y$_Z^\bullet$ re-reduction by Mn(II) is much faster at pH 7.5 than at pH 6.0 [34], while the rate of the Y$_Z^\bullet$ re-reduction by Q$_A^\bullet$ increases with decreasing pH [36,90,98]. The opposite pH dependencies for these two reactions may be explained by the difference in the source of the proton required for the re-reduction of Y$_Z^\bullet$; the proton is transferred from D1-His190 for Y$_Z^\bullet$ Q$_A^\bullet$ recombination but from the water molecule for Mn$^{2+}$ oxidation. Lower pH
promotes proton donation from D1-His190 by facilitating the protonated D1-His190, of which the pKₐ value has been estimated to be 6.9–8.3 in Mn-depleted PS II [74,76,82,91]. However, lower pH suppresses deprotonation of the water molecule bound to Mn(II).

4.4. Step 4

Protein conformational changes induced by the first photochemical event produce another binding site for Mn^{2+} in the apo-OEC followed by binding of another Mn^{2+} as ([Mn(II)-OH]₂^{2+}). This results in the second intermediate state that is corresponding to ‘State C’ in the kinetic model shown in Fig. 1.

The distance between YZ and [Mn(III)-OH]₂^{2+} at the first binding site increases as a result of the protein conformational changes; otherwise [Mn(III)-OH]₂^{2+} will be forced to be reprotonated by the transfer of the phenolic proton when YZ is oxidized. However, the weakened interaction will make the [Mn(III)-OH]₂^{2+} species unstable and able to be reprotonated more easily. This may facilitate the reduction of Mn(III) and lead to the loss of the ‘State C’ population, which lowers the quantum yield for photoactivation.

4.5. Step 5

Upon the second photochemical event, [Mn(II)-OH]₂^{2+} bound to the new site is oxidized to form [Mn(III)-OH]₂^{2+} via the same metallo-radical process (from step 2 to step 3) for the oxidation of the first [Mn(II)-OH]₂^{2+}.

4.6. Step 6

A di-µ-oxo-bridged or a di-µ-hydroxo-bridged Mn dimer core of the (Mn)₄-cluster in OEC is formed by disproportionation of two [Mn(III)-OH]₂^{2+} molecules [99]. The (Mn)₄-cluster is, then, formed by the subsequent binding of two further Mn(II) ions. If the bound Mn ions cannot be oxidized in the dark, a possible oxidation state of the (Mn)₄-cluster thus formed will be Mn(II)₂-Mn(III)₂ or Mn(II)₁-Mn(IV). Since the oxidation state of the S₁ state Mn XANES (X-ray absorption near edge structure) spectrum [5,100,101] and the simulation of the S₂ state multiline ESR signal [102,103] (but see [104]), the (Mn)₄-cluster thus formed needs to be oxidized by at least three electrons to reach the S₀ state in the native (Mn)₄-cluster. A three-electron reducing (Mn)₄-cluster has been produced by incubating the sample membranes with reductant such as NH₂OH, NH₂NH₂ or hydroquinone [105,106]. Therefore, these types of cluster may be formed as a precursor of the native (Mn)₄-cluster even in the process of photoactivation.

Ca²⁺ is certainly required for photoactivation [107] but is not involved in the present model because the mode of Ca²⁺ action in the photoassembly of the (Mn)₄-cluster is still a matter of debate. It was inferred that the binding of Ca²⁺ to the apo-OEC is required for photoassembly of the (Mn)₄-cluster [107]. Alternatively, it was suggested that PS II can assemble the (Mn)₄-cluster in the absence of Ca²⁺, but acquires O₂ evolution capability by the subsequent binding of Ca²⁺ [19,108,109]. It was also proposed that Ca²⁺ suppresses inappropriate ligation of non-functional Mn₃⁺ thus raising the photoactivation yield [110]. Another possibility is that Ca²⁺ induces a binuclear Mn(II)₂ core structure in the dark as a precursor of the (Mn)₄-cluster [49] as well as protein conformational changes that allow stable binding of the Mn(II) ion in the second stable intermediate (State C in Fig. 1) [32]. Therefore, Ca²⁺ may be involved in step 4 and/or stabilization of the [Mn(III)-OH]₂^{2+} species.

The proposed metallo-radical mechanism for photoassembly of the (Mn)₄-cluster is a speculative model and is clearly over-simplified. However, this model suggests that the (Mn)₄-cluster can be assembled by a reaction mechanism that is largely compatible with that for water oxidation by the (Mn)₄-cluster, in which YZ serves as an acceptor of hydrogen from substrate water bound to the (Mn)₄-cluster [63–65], or as an electrostatic promoter as well as a hydrogen acceptor [82,83]. In photoactivation, YZ tyrosine and D1-His190 abstract a H-atom from water molecules coordinated with the Mn(II) ion leading to assembly of the (Mn)₄-cluster for O₂ evolution. Therefore, both the reactions are intrinsic characteristics of PS II and no special tool and/or reaction of PS II is required for the photoassembly of the (Mn)₄-cluster,
although other additional factors and reactions are certainly required for the chemistry of each reaction. This view is consistent with the finding that the (Mn)$_4$-cluster can be photoassembled in a PS II core complex devoid of CP43 [24]. In other words, the photoassembly of the (Mn)$_4$-cluster and the water oxidation are not independent reaction processes, but rather different stages of one sequential reaction in PS II; the (Mn)$_4$-cluster can be assembled only when PS II is ready for oxidizing water. In chloroplasts, the D1 protein turns over very rapidly in a light-dependent manner due to the light-induced damage of the protein (photoinhibition), and the damaged D1 protein is selectively degraded leaving other PS II proteins intact and replaced by a newly synthesized one [111]. Therefore, the metallo-radical mechanism proposed in the present study is favorable for minimizing photodamage on the donor side of PS II during the assembly of the (Mn)$_4$-cluster.

The metallo-radical mechanism proposed in the present paper will be experimentally evaluated by studying the effects of H/D exchange and pH on the rate of electron transfer from the bound Mn$^{2+}$ to Y$_{ox}$ and on the kinetics of photoactivation. Measurements of proton release kinetics upon Mn$^{2+}$ oxidation and in each process of photoactivation may also provide useful information.

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