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A Role for Manganese in Oxygen Evolution in Photosynthesis

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The end of the petroleum era is at hand. The prospects are shrinking rapidly for a future for society based on liquid hydrocarbons as a major source of energy. Among the wide array of alternative sources that are currently undergoing scrutiny, much attention is attracted to the photolysis of water to produce hydrogen and oxygen gases. Water, the starting material, does not suffer from lack of abundance, and there is every likelihood that the environmental consequences of water splitting will be negligible.

Solar radiation is the obvious candidate for the ultimate energy source, but of course water cannot be photolyzed directly by the relatively low-energy wavelengths, greater than 300 nm, that penetrate the earth's atmosphere. Nevertheless, the photolysis of water to produce O_2 and reduced substances, with reduction potentials equivalent to that of H_2, is accomplished efficiently using sunlight by higher plant photosynthesis.[1] There are even organisms that, under special conditions, will evolve H_2 gas photosynthetically,[2] but not efficiently when coupled with O_2 production.

To produce a molecule of O_2 from water requires the removal of four electrons from two H_2O molecules.

$$2H_2O(liq) \rightarrow O_2(g) + 4H^+(aq) + 4e^-$$

If the electrons and H^+ ions combine, we can complete the reaction by

$$4H^+(aq) + 4e^- \rightarrow 2H_2(g)$$

In plant photosynthesis, however, the electrons are normally captured by low-potential electron acceptors like ferredoxin or NADP (nicotinamide adenine dinucleotide phosphate). Nevertheless, the energetics is very similar to that for the reactions above. For a survey of the energetics of photosynthesis, see the recent review by Blankenship and Parson.[3]

The value of $\Delta G°$ for the overall reaction of electron transport from water corresponds to 114 kcal (mol of $O_2)^{-1}$ or about 1.23 eV/electron transferred. In plant photosynthesis the electrons are transferred from water one at a time, and the photons absorbed by chlorophyll and the other photosynthetic pigments have energies of at least 1.8 eV. Despite the fact that there would seem to be enough energy per photon to transfer an electron, plants have evolved a scheme whereby this is accomplished by two light reactions acting in series. A current view of the Z scheme of photosynthetic electron transport is shown in Figure 1. Thus, the overall energy efficiency is no greater than 1.23/2(1.8) or 34%, even under optimal conditions, and the quantum requirement is at least 8 photons absorbed per O_2 evolved. While these figures may be modest compared with "optimum" values that one might set as a desirable target, they are nevertheless very appealing to workers in the field of solar energy conversion, where a 20% efficiency often seems to be barely within reach.

A key feature of the success of photosynthetic organisms is their ability to transfer electrons singly and store oxidizing equivalents that can be accumulated to oxidize water. Without this ability, even two light reactions in series would require 2.46-eV photons (λ = 500 nm) to provide the minimum energy necessary to split water. Furthermore, the second law of thermodynamics requires the loss of a significant fraction, perhaps 20%, of the photon energy for any practical solar converter,[4,5] and this would increase the minimum photon energy to 3.3 eV (λ = 375 nm). There is very little solar energy incident on the surface of the earth at wavelengths shorter than 375 nm.

On the other hand, there are difficulties associated with the storage of the intermediates produced by the one-electron oxidations. The standard reduction potential necessary to oxidize water to molecular oxygen is 0.82 V at pH 7. (Although the effective pH at the site of O_2 evolution in chloroplasts is not precisely known, it is probably not far from 7.) This means that the *average* reduction potential for the three intermediates involved must be about 0.8 V, and in some cases may exceed 1.0 V. The problem of stabilizing such

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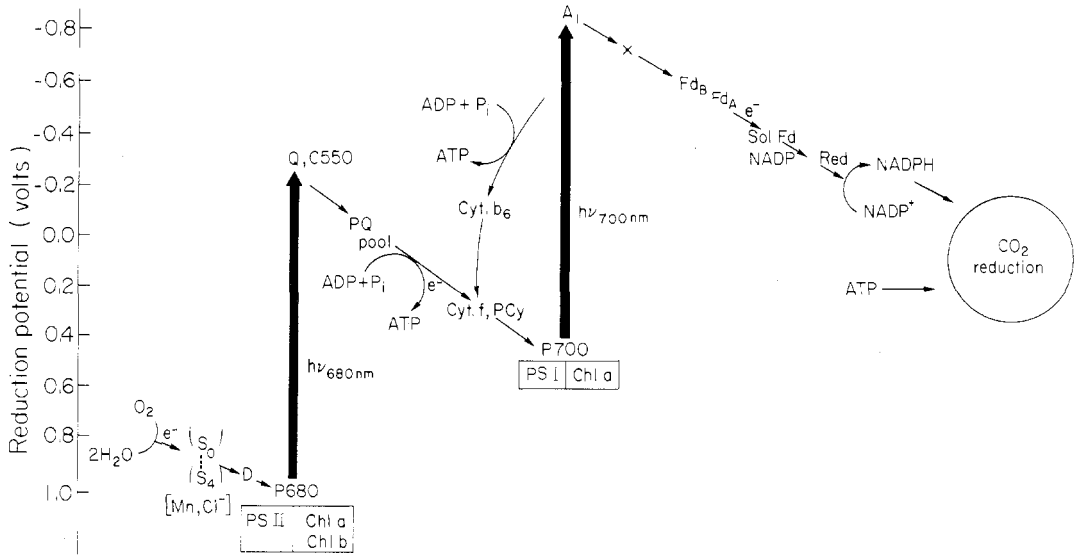

Figure 1. The Z scheme of electron transport for higher plant photosynthesis. The two light reactions of photosystem 1 and photosystem 2 are thought to operate in series, connected by a portion of the electron-transport chain involving quinones (Q, PQ), cytochrome *f* (Cyt *f*), and the copper protein, plastocyanin (PCy). Strong oxidants produced by photosystem 2 remove electrons from the Mn-containing complex that results in water oxidation. Photosystem 1 produces powerful reductants that donate electrons to ferredoxin (Fd) and NADP and are ultimately responsible for CO_2 reduction.

reactive and potentially oxidizing intermediates for extended periods (typically minutes) within close proximity (a few nanometers) of water is formidable. We are just beginning to understand how this is done, and the emerging story is a fascinating one.

Manganese Requirement for O_2 Evolution

For many years it has been known that oxygen-evolving photosynthetic organisms have a requirement for manganese.[1,6] Depletion of manganese in plants or algae by withholding it from the growth medium leads to the loss of O_2 evolution capability.[7] However, the activity can be restored within 0.5 h upon readdition of Mn^{2+} to the growth medium. Various experiments point to a site on the donor side of photosystem 2 as the location for the manganese requirement.[8,9] To date it has proved impossible to detect directly in the photosynthetic membranes the Mn-containing entity that is responsible for mediating O_2 evolution.

Manganese occurs in several pools in higher plant or algal cells. A portion of the complement of manganese occurs as Mn^{2+}(aqueous) in cytoplasmic or stromal fluids, presumably in equilibrium with a reservoir of Mn^{2+} that is weakly membrane bound. This portion, whose biological function is unknown, gives rise to an EPR signal characteristic of hexaaquomanganese(2+), and it can be removed essentially completely by cell rupture and washing of the pigmented membranes with chelating agents such as EDTA[10,11] (Figure 2, top spectrum). These washed chloroplast membrane preparations still have a high level of O_2 evolution from water in the Hill reaction, where artificial electron acceptors such as ferricyanide or indophenol dyes are used. The washed membranes also retain a portion of bound manganese that is EPR silent and corresponds

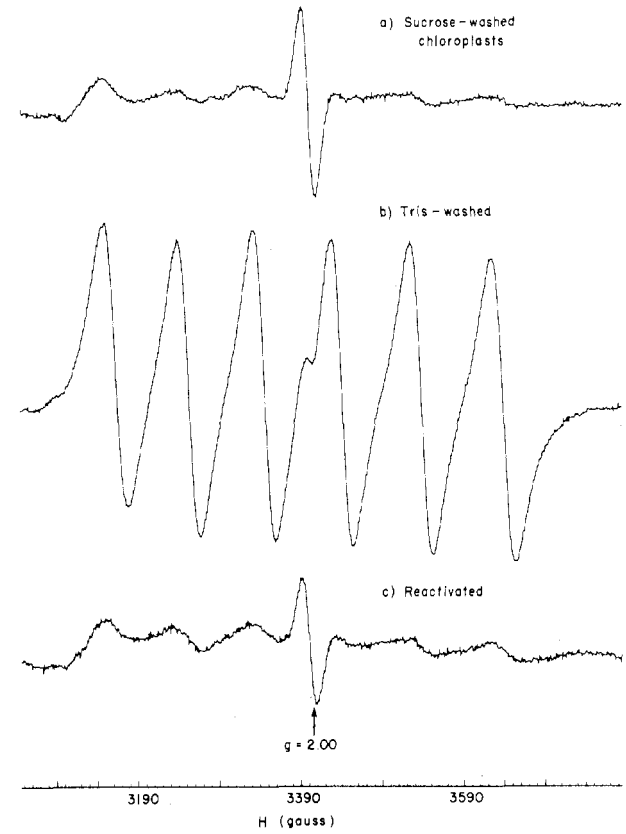

Figure 2. Chloroplast membrane fragments examined by EPR spectroscopy. (a) Washing with sucrose buffer containing chelating agents like EDTA leaves a very weak residual Mn signal (six hyperfine components) superimposed on a sharp signal near $g = 2.00$ (dark signal II). (b) Washing the above membranes with alkaline Tris buffer (0.8 M, pH 8.0) abolishes O_2 evolution and releases Mn^{2+}(aq) that exhibits a large-amplitude characteristic EPR signal. The Mn^{2+}(aq) is known to be physically trapped inside the closed thylakoid membranes in an aqueous inner space.[10] (c) Reactivation of O_2 evolution leads to a disappearance of the EPR signal of Mn^{2+}. (Figure taken from Blankenship et al.[11])

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(11) R. E. Blankenship, G. T. Babcock, and K. Sauer, *Biochim. Biophys. Acta*, **387**, 165–175 (1975).

to between six and eight atoms per photosynthetic electron-transport chain.[1,11]

Elegant experiments by Cheniae and Martin showed that this bound Mn consists of two portions.[9] About two-thirds can be released by treatments such as alkaline Tris washing or hydroxylamine extraction, and this release correlates with a loss of Hill reaction activity and inhibition of O_2 evolution. The remaining one-third of the Mn is very tightly bound, and its presence is not correlated in any obvious way with O_2 evolution. Other treatments that lead to the loss of O_2 evolution capability and/or membrane bound Mn, including mild heat treatment,[12] the use of chaotropic agents,[13] guanidine-HCl,[12] or exchange against high concentrations of Mg^{2+} (0.2 M),[14] are suggestive of the denaturation of a membrane-bound Mn complex.

Light is required along with Mn^{2+} to develop O_2 evolution capability in Mn-deficient algae.[4,15] When chloroplasts are inactivated by Tris washing, alternative donors to photosystem 2 can be used to provide electrons to $NADP^+$ or other electron acceptors.[16] In the presence of reducing agents and Mn^{2+}, the O_2 evolution reaction can be largely restored.[11,17] Using EPR detection, we have been able to show that Mn released from the chloroplast membranes by Tris washing is trapped as Mn^{2+} in the aqueous inner compartment of the thylakoids.[10] It then becomes bound into a membrane site again when O_2 evolution is restored.[11] The appearance of the characteristic six-line EPR spectrum of Mn^{2+}(aq) upon release from the membrane sites and the disappearance of this signal upon restoration of O_2 evolution are illustrated in Figure 2. Clearly, little irreversible denaturation of the binding site occurs during this process.

Manganese has been attractive as an element implicated in the water-splitting reactions of photosynthesis because of its multiple oxidation states, some of which involve relatively high reduction potentials. The source of the oxidizing power is in the photosynthetic electron-transport chain, more specifically, in the reaction centers of photosystem 2. Each photon that activates photosystem 2 leads to the transfer of a single electron from the water-splitting complex to the intermediate electron carriers, and a second photon entering photosystem 1 transmits the electron to the terminal acceptors in intact chloroplasts. Thus, each O_2 evolved requires the absorption of four photons in photosystem 2 and four photons in photosystem 1. The Z-scheme model implies an overall minimum quantum requirement of eight photons per O_2 evolved, which is in good agreement with a large body of experimental findings.[18]

Kok's S-State Scheme

Because photons arrive in a statistical fashion, not in groups of four or eight, and because the quantum efficiency of the Hill reaction remains high even to very low light fluxes,[19] there is an implication that relatively long-lived intermediates are formed and serve to store oxidizing intermediates in the water-to-O_2 path. Direct evidence for such intermediates appeared as a consequence of experiments initiated by Joliot and coworkers[20] and extended and interpreted by Kok and his associates.[21] They applied a train of brief (10 μs) saturating flashes of light to chloroplasts or O_2-evolving algae initially in the dark. Significant O_2 yields appear only after the third flash, and subsequent flashes in the train produce further O_2 pulses whose amplitude oscillates with a period of four flashes (Figure 3). After 25–30 flashes the oscillations damp out to give a uniform steady-state yield.

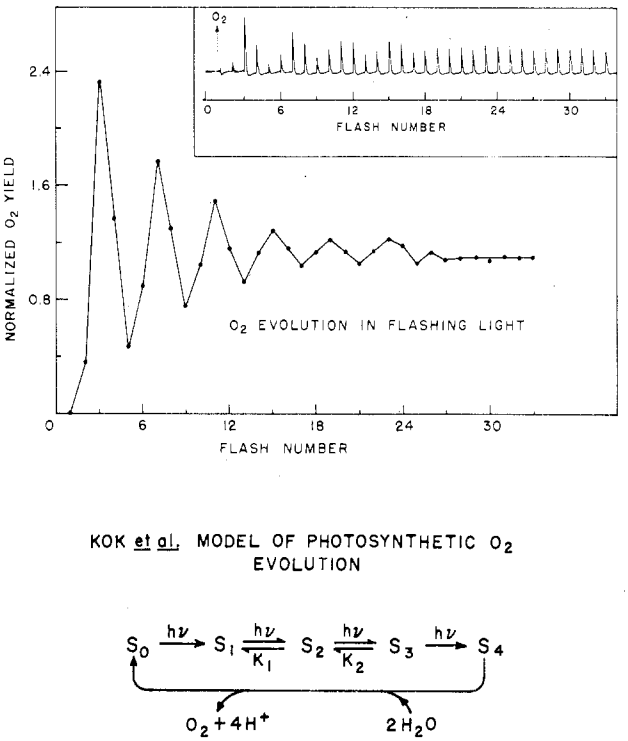

Figure 3. Period 4 oscillations in the yield of O_2 produced by a train of saturating, brief (10 μs) flashes illuminating a chloroplast sample. Inset shows the actual polarographic trace recorded from an oxygen electrode. (Taken from G. T. Babcock, Ph.D. Thesis, University of California, Berkeley, Sept 1973.) Below the figure is an early, simplified version of the Kok et al.[21] S-state model for accounting for the oscillations.

The most successful interpretation of the flash-induced O_2 yield oscillations was Kok's S-state scheme[21] that proposed a set of five states, S_0 through S_4, of unspecified molecular nature, representing successive stages of oxidation, or advancement, of the O_2 evolution complex.[22] Figure 3 shows a simplified form of the S-state hypothesis. To account for the high yield of O_2 on the third flash, Kok et al. proposed that S_1 as well as S_0 is stable in the dark, and that they are normally present initially in the ratio $S_1/S_0 = 3$. Occasional double hits (5–10%) and misses (10%) result in dephasing of the array of S-state complexes in a macro-

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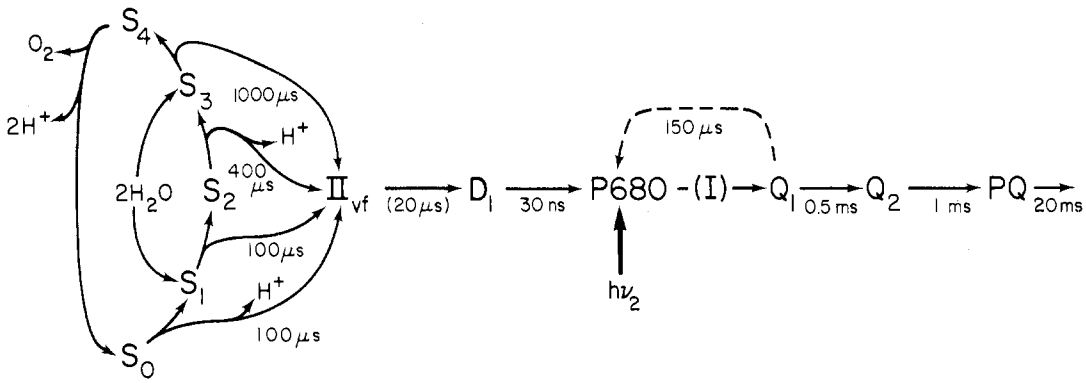

Figure 4. Photosystem 2 electron-transport components. Electrons are extracted one at a time from the S-state complex to advance it by stages through its cycle. A probable pattern of H^+ release is indicated along with the O_2-release stage. Discussion of the electron carriers and the kinetic constants given has been reviewed recently.[1,6,32]

scopic sample, and the oscillations soon damp out as a consequence. Although S_2 and S_3 are presumably powerful oxidants or species involving partially oxidized water, they are nevertheless stable for periods of the order of minutes at room temperature. They relax back to S_1 if no further photon activation occurs during this interval (Figure 3, scheme).

Attempts to identify the molecular nature of the S-state components were not highly rewarding. Until recently, there have been no reports of associated optical absorption changes, EPR signals, or other direct physical measurements, despite extensive efforts and appreciable sensitivity for detection. There is no support for the proposal that Mn incorporated in a porphyrin, perhaps manganese chlorophyll, is involved in the water-splitting complex. Manganese porphyrins have moderately strong charge-transfer bands in addition to the porphyrin π–π^* transitions in the visible spectrum, and the charge-transfer components change significantly with both oxidation state and axial ligation at the metal.[23] No changes of this type can be detected in vivo in sensitive absorption transient studies.

Manganese in the S-State Complex

Indirect approaches to characterizing the S states have achieved some measure of success. By monitoring proton magnetic resonance relaxation of solvent water protons, Wydrzynski et al. were able to demonstrate that paramagnetic manganese, presumably Mn(II), in chloroplast membranes can increase significantly the proton spin-relaxation rates.[24] In experiments where a group of flashes (0 to 20 flashes) was given to spinach chloroplasts prior to measurement, the relaxation rates exhibited oscillations with a period of 4 flashes.[25] Analysis of these results in terms of the S-state hypothesis led to the surprising conclusion that the oxidation state of Mn in the complex does not increase progressively from S_0 through S_3. In particular, the Mn in S_3 is significantly less oxidized than in S_2.[26] It is difficult to calibrate these measurements in quantitative terms, however, and the stoichiometric aspects of the oxidation state changes are not determined easily by this method.

Govindjee et al. proposed a mechanism to account for the Mn oxidation state changes associated with the S-state advance.[26] They postulated the participation of four Mn atoms per complex, with the following composition: S_0[2Mn(II),2Mn(III)] becomes oxidized to S_1[Mn(II),3Mn(III)]; but S_2 is also [Mn(II),3Mn(III)] in this proposal, and S_3 is actually more reduced, containing [3Mn(II),Mn(III)]. In the $S_1 \rightarrow S_2$ and $S_2 \rightarrow S_3$ transitions, it was proposed that the "missing" oxidizing equivalents reside in water, which has been incorporated into the complex in a partially oxidized form (OH, OH^+).

Proton Release

We shall return to the question of Mn oxidation state changes in connection with measurements made in a different way in our laboratory, but first let us examine the flash-number behavior of the release of H^+, the other detectable product of the water-splitting reaction. Clearly, if the transition $S_3 \rightarrow S_4 \rightarrow S_0$ involves a concerted four-electron oxidation of two water molecules, then four protons should be released at the same time, as suggested in the early scheme of Kok et al.[21] (Figure 3). The experimental measurements show that this does not happen. Although the detection of proton release associated with water oxidation is complicated by other proton translocations across the thylakoid membranes that need to be subtracted out,[27,28] each of the three groups that has carried out such studies agrees that the concerted process cannot be correct.[28-30] For the present the data do not justify going beyond the simplest whole-number values for the proton release pattern. Even so, there remains a disagreement between Fowler[28] and Saphon and Crofts,[29] on the one hand, who believe that the pattern is 1,0,1,2 for the number of protons released, respectively in the steps $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$, and $S_3 \rightarrow [S_4] \rightarrow S_0$, and Junge et al.,[30,31] who believe that the pattern is 0,1,1,2. The experimental results of these groups clearly differ, especially with respect to the release of protons following the first flash and the amplitude of the oscillations of period 4. Further investigations of these dif-

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ficult measurements are needed. A scheme showing the removal of electrons one at a time[32] by photosystem 2 and incorporating the proton release pattern of Fowler and of Saphon and Crofts is shown in Figure 4.

Evidently water participates in different ways in the four steps, even allowing for the disagreement in the reports of the behavior of the first two steps. However, these results alone do not permit us to distinguish among several possibilities with respect to the mechanistic origin of proton release. For example, one source could be the dissociation of a water molecule to provide a hydroxyl anion, OH^-, to compensate for an increased positive charge of the Mn centers in the state S_{n+1} relative to S_n. Another origin could lie in the partial oxidation of one water to the level of peroxide, ·OH, or two waters to the level of hydroperoxide, ·OOH, or superoxide, $\cdot O_2^-$, that is incorporated into the S-state complex.

Manganese Oxidation State Changes

In experiments conducted by Dr. Tom Wydrzynski in our laboratory at Berkeley, we tried a different approach to evaluate the participation of manganese more precisely and more quantitatively. We knew that Mn bound into the chloroplast membranes is undetectable using standard EPR techniques, but that it could be seen readily upon release by various techniques that inactivate the water-splitting complex.[10,13] Furthermore, we knew that only Mn^{2+} can be seen in this way; the method is insensitive to Mn(III), MnO_2, or other higher oxidation-state species. Preliminary studies quickly showed that the Mn^{2+}(aq) EPR signal detected after a brief heat treatment (2 min at 55 °C) of chloroplasts is decreased in amplitude (relative to a dark, heat-treated control) when the chloroplasts are illuminated just prior to heating.[33] Furthermore, the light-stimulated decrease in Mn^{2+} release is abolished by the classical inhibitors of O_2 evolution, dichlorophenyldimethylurea or fluorocarbonyl cyanide phenylhydrazone. The decrease in EPR signal amplitude also disappears progressively ($t_{1/2}$ = 40 s) when we include a dark interval between the end of the illumination period and the start of the heat treatment. This corresponds to the known lifetime of the states S_2 and S_3.[21,22]

What is the fate of the "missing" Mn in the illuminated chloroplasts? Is it still bound into the membrane, or is it released in a higher oxidation state that is undetectable by EPR? We know that Mn^{3+} is unstable in aqueous solution and, if released in that oxidation state, it should undergo rapid disproportionation by the reaction

$$2Mn^{3+} + 2H_2O \rightarrow Mn^{2+} + MnO_2 + 4H^+$$

Any Mn(IV) present in the membrane complex prior to heating should appear directly as MnO_2 upon release, assuming that no reducing substances from the membrane reach it first.

To look for the presence of MnO_2 in our heat-treated samples we added H_2O_2 (0.6% in the final mixture), a reagent that reduces MnO_2 readily in slightly acidic solutions.

$$H_2O_2 + MnO_2 + 2H^+ \rightarrow Mn^{2+} + O_2 + 2H_2O$$

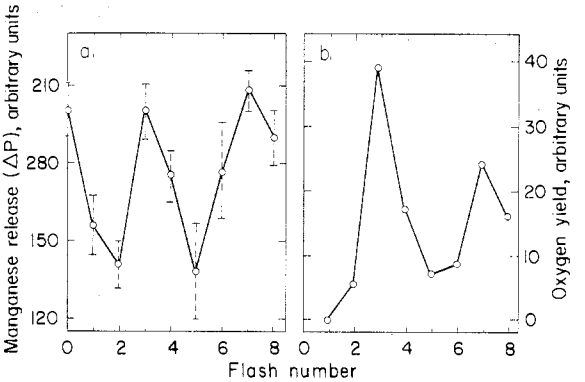

Figure 5. Illumination with a group (n = 0–8) of saturating flashes produces oscillations in O_2 yield (right) or in the yield of EPR-detectable Mn^{2+}(aq) released by a heat treatment (5 min at 55 °C) of the chloroplasts (left). (Taken from Wydrzynski and Sauer.[33])

When we applied this treatment to illuminated, heat-treated chloroplasts, the addition of H_2O_2 following heat treatment increased the amplitude of the Mn^{2+}(aq) EPR signal to the level of the dark control. Furthermore, the reduction by H_2O_2 occurs when the medium is buffered at pH 6.0 but not at pH 7.5, indicating that MnO_2 is the form in which the undetected manganese occurs. From these experiments we conclude that we have uncovered a quantitative assay for the degree of oxidation of the manganese associated with photosynthetic oxygen evolution. In other words, following the heat inactivation the released Mn retains a memory of its oxidation state prior to inactivation.

We then applied a group of light flashes to monitor the oxidation state changes associated with each of the S states. By preilluminating chloroplast samples with up to eight saturating flashes (20-μs duration) at 4-s intervals prior to heat treatment, we observe oscillations of period 4 in the amount of released Mn^{2+} detected by EPR (Figure 5). As expected, the oscillations are abolished if H_2O_2 at pH 6.0 is added after the heat treatment. Furthermore, the phase of the oscillations corresponds rather closely to that of the proton spin relaxation rate changes that had been seen earlier.[25,26]

To interpret our findings quantitatively in terms of the oxidation state of manganese in each of the states S_0 through S_3, we made the following assumptions: manganese present in the complex as Mn(II) prior to release is detected directly and quantitatively as Mn^{2+} by the EPR assay; Mn(III) undergoes disproportionation to give equal amounts of Mn^{2+} and MnO_2; Mn(IV) is converted directly to MnO_2 upon release and is not detected unless a post-addition of H_2O_2 is provided; the advance of the S states with flash number proceeds according to the Kok scheme.[21] (We also examined an alternative scheme proposed by Thibault,[34] but it does not give better agreement with our results than the Kok scheme does. From other experimental tests, we have reason to prefer the Kok scheme.)

With the above assumptions we first determined from the pattern of O_2 evolution from our chloroplasts the best values for the Kok parameters for the initial ratio (S_1/S_0), 2.90, the miss parameter α, 0.103, and the double-hit parameter β, 0.100, for untreated chloroplasts. With these values we were able to calculate the

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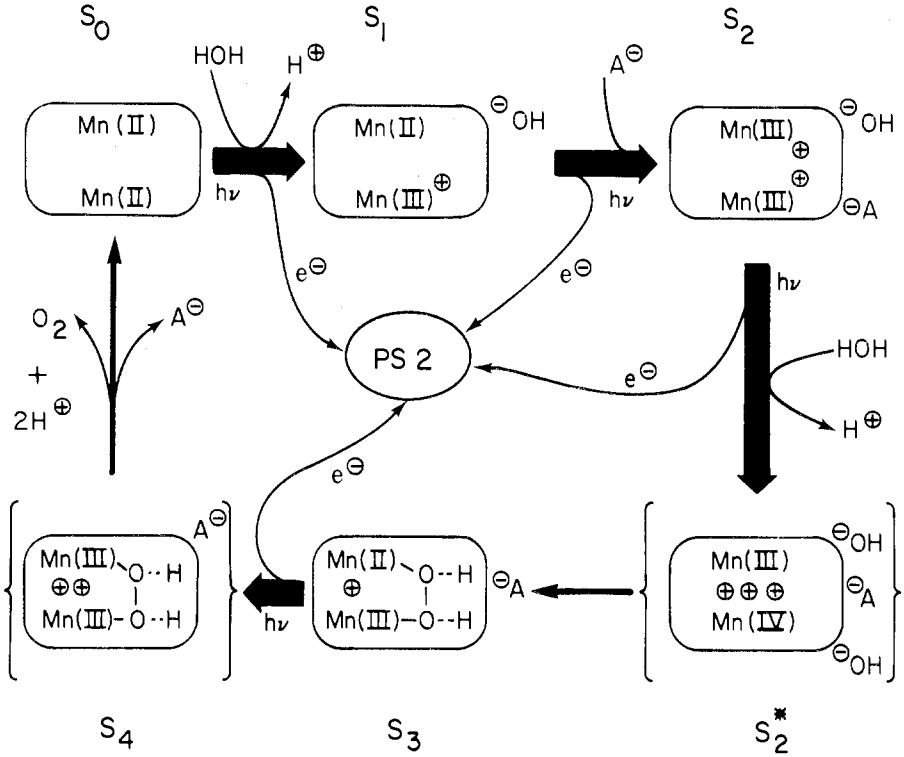

Figure 6. A proposal for the role of Mn in water oxidation in photosynthesis based on a mechanism presented by Wydrzynski and Sauer.[33] States S_0, S_1, S_2, and S_3 are sufficiently stable to be detected in Mn-release studies. S_2^* and S_4 are hypothetical intermediates for which there is no direct evidence. Binding of OH^- results in release of H^+ whereas binding of A^- (probably chloride ion) does not. The existence of an O–O bond in S_3 is not known.

relative amount of each S state initially in the dark and then after each flash. For example, in the dark S_0 = 0.256 and S_1 = 0.744; after one flash S_0 = 0.026, S_1 = 0.280, S_2 = 0.619, S_3 = 0.074; and so on. Next, we assign a weighting factor to describe the Mn(II) character of each S state and solve the (overdetermined) set of equations describing the Mn^{2+} EPR signal amplitude in the dark and after each of the first eight flashes. The entire procedure was repeated for a second set of experiments where the addition of 0.5 mM $K_3Fe(CN)_6$ to the intact chloroplasts in the dark increased the initial ratio (S_1/S_0) to 5.21 and slightly increased the α parameter. The average of these two sets of Mn(II) weighting factors, normalized to S_0 = 1.00, is S_1 = 0.68 ± 0.03, S_2 = 0.58 ± 0.03, and S_3 = 0.78 ± 0.14.

The weighting factors can now be compared with the values predicted for particular models. For example, if only one Mn atom is present in each O_2 evolving complex and it occurs as Mn(II) in S_0 (giving a weighting factor of 1.00), then a one-electron oxidization will produce Mn(III) in S_1. The disproportionation of Mn(III) upon release yields half as much Mn^{2+} as does Mn(II). A second one-electron oxidation would give Mn(IV) and produce a weighting factor of 0.0 for S_2. Any one-electron reduction step would increase the weighting factor by 0.5. These conclusions are clearly at variance with the calculated values above, where the step sizes are significantly smaller. This evidence leads us to rule out a complex containing only one manganese atom.

If we consider a complex containing two Mn atoms, then we can generate the set of weighting factors 1.00, 0.75, 0.50, 0.75 by assuming that S_0 contains $Mn^{II}Mn^{II}$, S_1 contains $Mn^{II}Mn^{III}$, S_2 contains $Mn^{III}Mn^{III}$, and S_3 is in the less oxidized state $Mn^{II}Mn^{III}$. This is illustrated in the mechanism shown in Figure 6, which also incorporates features to account for the pattern of H^+ release. We shall return to this presently. The predicted weighting factors for this mechanism agree well with the determined values,[33] certainly within reasonable experimental error. (In principle, the Mn-release experiments cannot distinguish between $Mn^{III}Mn^{III}$ and $Mn^{II}Mn^{IV}$ for S_2; however, the effect of these two possibilities on proton magnetic resonance relaxation would be quite different, because only Mn^{II} should be an efficient relaxer. Those studies clearly support the $Mn^{III}Mn^{III}$ assignment.[25])

If the complex contained more than two Mn's in a cooperating unit, as suggested by some analytical data and proposed in several previous models,[26,35,36] then the step sizes for the weighting factor changes would be less than what we see, at least assuming that the complex is fully reduced in S_0. The particular model proposed by Govindjee et al.,[26] for example, involves four cooperating Mn and has an S_0 state that is not fully reduced. Using our approach for their model, one would predict weighting factors of 0.75, 0.625, 0.625, 0.875 for S_0, S_1, S_2, and S_3, respectively. These will not fit our observations, even if they are renormalized.

Models for the Water Splitting Complex

Renger has proposed a model for the water-splitting complex that is in better accord with the available experimental observations.[37] It postulates a binuclear Mn complex where water ligands are the source of an O–O bond at the level of bound peroxide that is binuclearly

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complexed. Besides the functional manganese, he postulates an additional donor that can undergo a one-electron oxidation at moderate redox potential to a species that is stable in the dark (e.g., in state S_1). This oxidation is not coupled to deprotonation in Renger's mechanism, although their experimental proton release measurements[30] are at variance in this regard with those of Fowler and of Saphon and Crofts.[29] Subsequently, molecular oxygen is formed as a binuclearly complexed species when this oxidized donor removes an electron from a complexed superoxide. The exergonic release of O_2 is accomplished by replacement with fresh water ligands. Appealing features of this model include the avoidance of high reduction potential species that would be difficult to stabilize, the formation of the O–O bond in a binuclear complex involving partial water oxidation, the release of protons in a pattern that is compatible with experiments, and intermediate states that achieve the water oxidation by steps that involve successive one-electron removal and implicate intermediates that may reasonably be expected to be stable.

To bring Renger's model into agreement with the manganese data it is necessary to propose that the "one-electron donor" is, in fact, a part or aspect of the manganese complex and involves the conversion of Mn(II) to Mn(III). Proton release on the $S_0 \rightarrow S_1$ transition can be accommodated by postulating the association of a hydroxide ion derived from water bound at the Mn(III) site and neutralizing the increased positive charge of S_1. The absence of proton release for $S_1 \rightarrow S_2$ may mean that charge neutralization is accomplished by binding a permanent anion, such as Cl^-. There is a well-documented requirement for an anion like Cl^- to enable the water splitting enzyme to function.[38,39]

A mechanism that we have developed to accommodate the most recent findings is summarized in Figure 6. It envisions two Mn atoms associated in a single complex with ligands derived from protein amino acid side chains or from water. The most reduced state, S_0, has both Mn atoms in the +2 oxidation state. Removal of one electron by the photosystem 2 light reaction increases the positive charge on the metal atoms in the complex; the metal atoms then coordinate OH^- from water and release a proton. This state, S_1, is also stable in the dark. Removal of a second electron increases the oxidation state to III,III, but no proton is released. Because the positive charge on the Mn complex has presumably been increased in forming S_2, the counterion must be obtained without dissociating water. Removal of the third electron decreases the oxidation state to II,III, most likely by transferring two electrons from bound oxygen-containing ligand(s). The release of a single proton could be a consequence of binding a second OH^- prior to electron relocalization (or possibly from the formation of an oxide). At the present we do not know whether the O–O bond has formed in S_3. If a binuclear Mn, peroxo-bridged or superoxo-bridged complex is present, then the two protons shown in S_3 in Figure 6 would have to be transferred to other binding sites in the complex. They are released only upon removal of the fourth electron to achieve state S_4, which then releases O_2 and returns the complex to state S_0.

Recent Developments

Two recent findings support the model shown in Figure 6. Spector and Winget have succeeded in extracting a colorless, manganese-containing protein from chloroplast membranes that has the characteristics of the water-splitting enzyme.[40] The chlorophyll-containing membranes that have been depleted by this extraction are incompetent in the Hill reaction, but competence can be restored by reconstituting the depleted membranes with the purified Mn–protein complex in lipid vesicles. The protein contains 2 Mn/65 kdalton of peptide. (It can also be extracted under certain conditions in a dimeric form; however, the monomeric complex seems to be the active form in reconstitution studies.) Upon treatment with alkaline Tris buffer, the isolated protein releases Mn^{2+}, just as do the intact membranes. This research is a very exciting, new development in the exploration of the mechanism of water splitting, and it is already stimulating a wave of investigations of the properties of the Mn–protein complex.

Drs. Melvin Klein and Jon Kirby and co-workers in our laboratory at Berkeley have succeeded in monitoring the membrane-bound Mn in chloroplasts using X-ray absorption edge fine structure spectroscopy (XAEFS) and extended X-ray absorption fine structure (EXAFS). These approaches, which make use of synchrotron radiation from the electron storage ring at the Stanford Linear Accelerator, permit one to deduce information about the oxidation/coordination state of elements like Mn using spectroscopy involving inner (core) electrons of the atoms.[41] Preliminary results have been obtained and two significant conclusions are apparent.[42] The oxidation state of the Mn bound in chloroplast membranes is confirmed to be relatively low (in the range of II to III), but it is distinctly higher than that of Mn^{2+}(aq) obtained upon release by Tris washing. Of even greater significance are the results of the EXAFS studies that lead to the conclusion that each Mn atom has, as a near neighbor, a second transition-metal element that could very well be another Mn. The distance of separation is 2.70 Å, which is quite comparable with known binuclear Mn di-μ-oxo model compounds with two O atom bridges.[43]

Conclusion

Although the role of manganese in the water-splitting reaction leading to oxygen evolution in photosynthesis now seems definitely to be established, the detailed structure of the complex and the mechanism of its action remain to be determined. The complex appears to contain two Mn atoms per active site which are in close proximity to one another. Oxidation state changes involving Mn(II), Mn(III), and probably Mn(IV) are implicated, and at least one intermediate species ap-

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(42) J. Kirby, A. S. Robertson, J. P. Smith, A. C. Thompson, D. B. Goodin, and M. Klein, to be submitted.
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pears to involve a bound form of partially oxidized water. The nature of the strong oxidant (high potential electron carrier) that removes electrons one at a time from the complex is not yet known; however, a chlorophyll monomer has been proposed[44] as a candidate for the primary donor of the reaction center of photosystem 2, and cytochrome *b*-559 in some special high-potential form may serve to mediate electrons between it and the water-splitting complex.

The pioneering mechanism proposed by Bessel Kok to account for the periodic oscillations in O_2 flash yields is still the best framework for seeking an understanding of this process. Most of what we have learned in the 10 years since it was first presented can be incorporated easily into his overall scheme. As more detailed information becomes available we can expect to approach a better understanding of the molecular basis of the process. This will clearly be of importance in designing chemical model systems for simulating the photosynthetic mechanism that utilizes sunlight, water, and carbon dioxide to provide us with our best cheap source of available energy.

This Account is dedicated to the memory of Dr. Bessel Kok who made enormously fruitful contributions to our present understanding of photosynthetic electron transport and especially to the subject of this paper. I am indebted to my colleagues whose work is described in the text of this article. Much of this work was supported by the Divisions of Basic Energy Sciences and Biomedical and Environmental Research of the U. S. Department of Energy (Contract W-7405-ENG-48) and by a grant from the National Science Foundation (PCM 76-05074).

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Mechanisms of Flavin Catalysis

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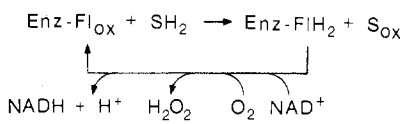
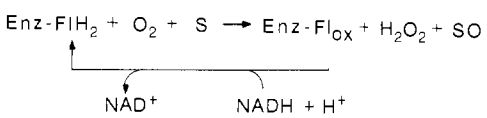
There are now recognized over 100 flavoproteins. Many contain more than one flavin, and others require the presence of Fe_nS_n cluster cores, exotic and fascinating inorganic cofactors of molybdenum, etc. (for examples, see ref 1). Flavoenzymes, for which flavin and substrate undergo a direct oxidation–reduction reaction, may, in the main, be placed in one of three categories: (i) the flavodoxins which are responsible for the transport of electrons by alternating between reduced and radical states; (ii) biological dehydrogenating agents (eq 1); and (iii) biological agents responsible for



(1)

the oxidation of substrate by the "activation" of molecular oxygen and transfer of one or two oxygen atoms from $^3O_2$ to the substrate (eq 2). We shall deal in this



(2)

Account with the mechanisms by which non-enzyme-bound flavins enter into the forward reactions of eq 1 and 2. The mechanistic deductions derived from these model studies are extrapolated to the mechanisms of the flavoenzymes.

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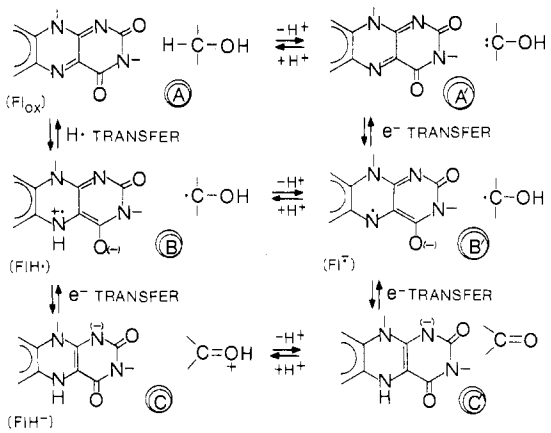
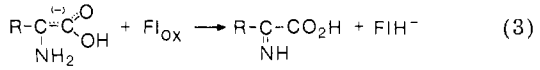
Scheme I

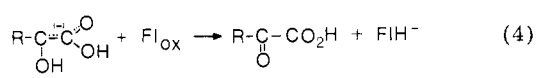


Dehydrogenation Reactions

Introduction of unsaturation α,β to a carboxyl group is performed by an important group of flavoenzymes. It has been proposed that the initial step is the ionization of the proton α to the carboxyl group followed by oxidation of the resultant carbanion (D- and L-amino acid oxidase (eq 3), lactic acid oxidase (eq 4), succinic



(3)



(4)

acid oxidase (eq 5), etc.). Evidence for the formation

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