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Oxidation of Water to Molecular Oxygen

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ABSTRACT

Numerous papers have been published on the topic of water oxidation; however, the light-driven water splitting enzyme still remains one of the enigmas of photosynthesis. In this chapter, we have summarized the advances made on the oxidation of H_2O to O_2. The water-oxidizing complex includes, at least, the reaction center proteins D1 and D2 and a 33 kilodalton extrinsic protein; other proteins seem also to be involved. The 33 kDa extrinsic protein may turn out to be dispensable for the molecular mechanism of O_2 evolution. Chloride and calcium ions are also required, although their exact functions remain unknown. It is clear, however, that Mn undergoes dynamic changes as the oxygen clock moves from relaxed states S_0 to S_1 and S_1 to S_2. This is followed by conversion of S_2 to S_3 and S_3 to S_4 until O_2 is evolved. It is accepted that Mn is the charge accumulator, but it is considered likely now that at one of the steps, a histidine residue may act as a redox active ligand, and store the charge. It was generally believed that Hs^+ are released as 1, 0, 1, 2 during $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$, and $S_3 \rightarrow (S_4) \rightarrow S_0$ transitions. However, the currently accepted pattern is 1, 0.5, 1 and 1.5. The nature of the intermediates of water oxidation form H_2O to O_2 is still unknown. Although the recent knowledge about the 3–D crystal structure of the reaction center complex from purple photosynthetic bacteria has led to a more precise picture of a portion of the water oxidizing complex than known before, further understanding will come after this complex is crystallized and its 3–D structure known and after methods are evolved to trap and monitor transient intermediates in water oxidation.

I. Introduction

Molecular oxygen (O_2) was nearly absent when the earth's atmosphere was formed approximately 4.5 billion years ago (Rao *et al.*, 1981; Wayne, 1988). Fossil evidence suggests that prokaryotes inhabited the earth as far back as 3.5 to 3.8 billion years ago. The appearance of significant levels of atmospheric O_2 approximately 2 billion years ago must have followed the rise of photosynthesis. It is considered highly likely that cyanobacterial photosynthesis may have been present much earlier (about 3.5 billion years ago) but O_2 did not accumulate in the atmosphere, either because various geochemical reactions were consuming it or because it was being used up by other aerobic forms of life that had also evolved (Veizer, 1988). Although it is conceivable that oxygen production was detrimental to many other organisms living at the time (since they were likely to have been anaerobic heterotrophs (Rao *et al.*, 1981; Cavalier–Smith, 1987)), terrestrial organisms ultimately benefited from this new development in at least two respects. For example, the availability of O_2 as a terminal electron acceptor made it feasible to generate adenosine triphosphate (ATP) by respiration. Likewise, the introduction of large amounts of O_2 into the earth's atmosphere must have helped to create the ozone layer in the lower stratosphere, which blocks out short-wavelength solar UV radiation (Turco, 1985; Wayne, 1988).

For photosynthetic organisms, the evolution of oxygenic photosynthesis was a major biochemical breakthrough: it enabled them to exploit a virtually unlimited source of electrons by oxidizing water. Water is an extremely poor reductant, however, and thus a system to remove electrons from such a substrate (where the energy input for each step is restricted to a quantum of visible light) must be efficient at generating a strong oxidant and must provide a "charge-accumulating" complex to handle the four oxidizing equivalents needed to oxidize two H_2O molecules to dioxygen:

$$2\ H_2O = O_2 + 4H^+ + 4e^-$$

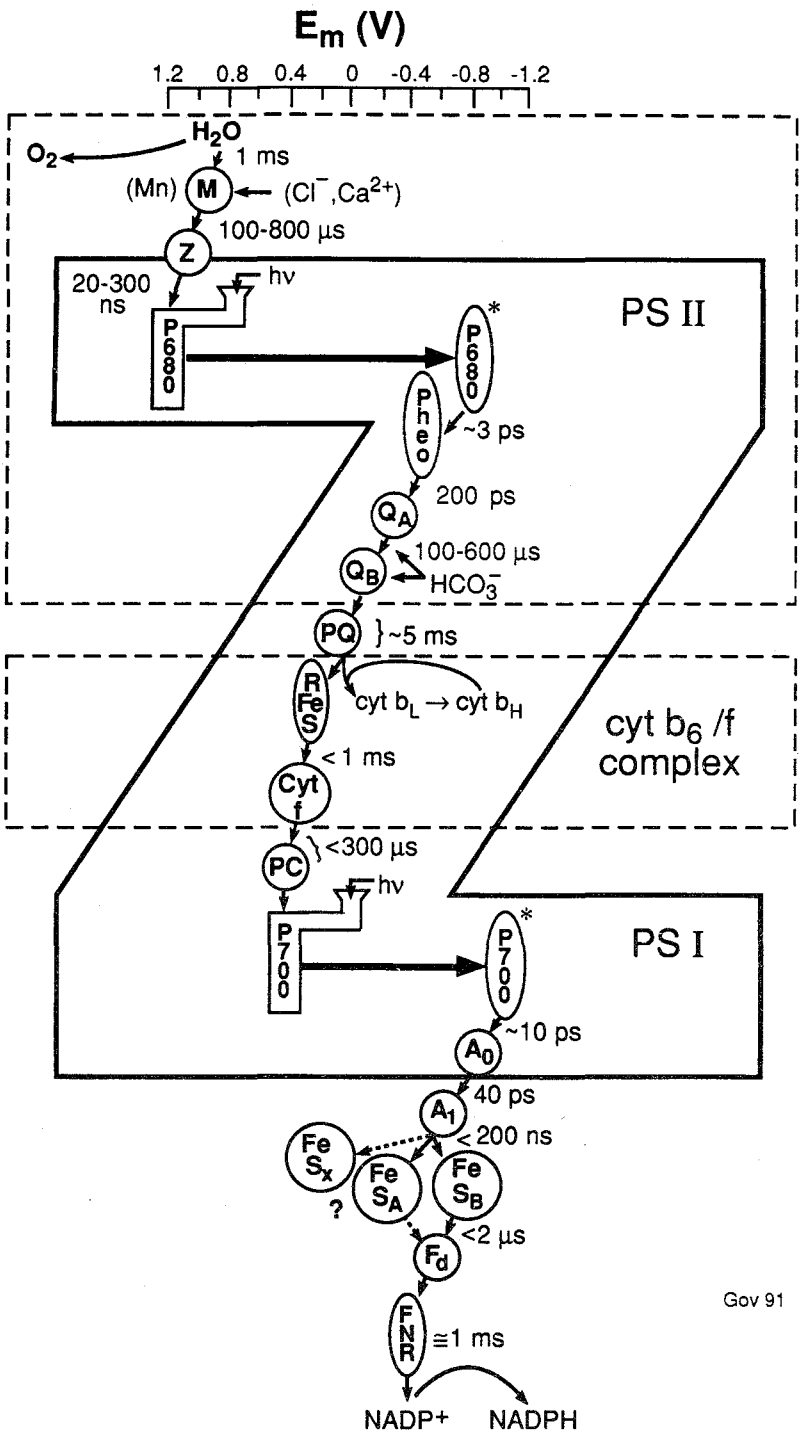
The photosynthetic reactions that generate molecular oxygen are part of a highly-ordered series of photochemical events that are coupled to two electron-transfer

mechanisms. The two mechanisms operate on opposite ends of a specialized pigment-protein complex, known as photosystem II (PS II) or the water-plastoquinol oxido-reductase, which is found within the cells of cyanobacteria, multicolored algae and higher green plants. One of these directly generates O_2 by removing four electrons and four protons from two water molecules. The splitting of water releases protons into the inner aqueous compartment of the thylakoid membrane. The other reduces plastoquinone (PQ) to plastoquinol (PQH_2), which is capable of shuttling two protons and two electrons. Another photosystem (PS I) transfers electrons from plastoquinol to nicotinamide dinucleotide phosphate ($NADP^+$), producing the NADPH required for the reduction of CO_2. On the other hand, ATP, also needed for CO_2 fixation, is synthesized by using the proton gradient generated by these mechanisms. Light absorption by chlorophylls (P680 and P700) at the heart of the two photosynthetic protein complexes (the reaction centers I and II) provides the energy to drive these two reactions (for details, see Fig. 1 and its legend). The molecular structure that accomplishes this feat is now being examined in detail.

For earlier reviews on photosystem II, see Govindjee (1980, 1982, 1984), Velthuys (1980, 1987), van Gorkom (1985), Diner (1986), Mathis (1986), Ort (1986) Andréasson and Vanngård (1988), Hansson and Wydrzynski (1990) Andersson and Styring (1991) and Vermaas and Ikeuchi (1991). The electron acceptor side of PS II has been discussed by Vermaas and Govindjee (1981), Crofts and Wraight (1983), Govindjee and Eaton-Rye (1986), and Govindjee and Wasielewski (1989), and the electron donor side by Ghanotakis and Yocum (1985), Govindjee *et al.* (1985), Renger and Govindjee (1985), Babcock (1987), Renger (1987a, b), Coleman and Govindjee (1987), Homann (1987), Brudvig *et al.* (1989), Rutherford (1989), Babcock *et al.* (1989) and Ghanotakis and Yocum (1990). Finally, the relation of the work on the reaction center of purple bacteria to photosystem II has been discussed by Michel and Deisenhofer (1988).

II. Proteins and Cofactors

The PS II complex is embedded within the lipid bilayer of the thylakoid membrane. There are three electron-transfer complexes in the membrane (Figs. 2 and 3): PS II (under discussion here), the cytochrome *bf* complex (which transfers electrons from PS II to PS I; see Cramer *et al.*, 1991), and PS I (Golbeck and Bryant, 1991). The current picture of PS II (Fig. 2) suggests that in additon to the reaction center protein, composed of polypeptides D1 and D2 (molecular masses 39 kilodaltons each) and cytochrome b-559 (a 4 and 9 kilodalton heme-containing protein whose function is unknown), there are also at least three polypeptides of molecular mass 33, 24 and 17 kilodaltons that are bound to the inner surface of the thylakoid membrane and that contribute to oxygen production in plants. A number of other recently discovered polypeptides (with a molecular mass between 4 and 20 kilodaltons) are associated with PS II, but their functions are still uncharacterized. Other chlorophyll-containing pigment proteins (CP-43, molecular mass 52 kilodaltons; and CP-47, molecular mass 56 kilodaltons) help to transfer excitation energy into the reaction center. Several organic and inorganic ions (iron, manganese, calcium, bicarbonate and chloride) are also involved in catalyzing electron transfer, maintaining the protein structure, or


Em (V)
1.2 0.8 0.4 0 -0.4 -0.8 -1.2
H2O
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(Mn)
M
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20-300 ns
hν
P680
P680*
PS II
Pheo
~3 ps
200 ps
QA
100-600 µs
QB
HCO3−
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}~5 ms
RFeS
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cyt b6 /f complex
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PC
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P700*
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regulating activity. Since cyanobacteria do not contain the extrinsic 17 and 24 kilodalton proteins and since a *Synechocystis* mutant lacking extrinsic 33 kilodalton polypeptide (L. Sherman, B. Zilinskas and others, personal communication) can carry out O_2 evolution, these 3 extrinsic polypeptides cannot be considered an absolute requirement for the O_2 evolution mechanism. For other details, see Andersson and Styring (1991) and Ghanotakis and Yocum (1990).

Due to the inherent complexity of PS II, a number of advances in our understanding of how a charge separation is accomplished have come from simpler biological systems. The simplest chlorophyll-containing photosynthetic complexes that have been extensively studied as models for PS II occur in the purple non-sulfur bacteria (for reviews, see Feher *et al.*, 1989; Coleman and Youvan, 1990). This bacterial system also catalyzes the reaction that is intrinsic to all such complexes, that is, converting light energy into a transmembrane electrochemical potential gradient (generated by a charge separation and the pumping of H^+). Energy stored in this gradient is subsequently used by the complex known as the ATP synthase or coupling factor to synthesize ATP for cellular metabolism (Ort and Melandri, 1982; Jagendorf *et al.*, 1991).

Figure 1. (on facing page): The linear photosynthetic electron transfer pathway in plants and cyanobacteria: the Z Scheme. The energy from two separate photosystems (I and II) connected in series is used to oxidize water and reduce $NADP^+$. Photysystem (PS) II, whose reaction center core partly resembles that of the purple bacteria, oxidizes two H_2O molecules into $O_2 + 4H^+ + 4e^-$ and reduces plastoquinone (PQ) to PQH_2 in a $2e^-/2H^+$ reaction. Photysystem (PS) I, which resembles the reaction center of certain green sulfur bacteria, oxidizes the electron-carrying molecule plastocyanin (PC) and reduces $NADP^+$ to NADPH. The cytochrome b_6f complex functions analogously to its bacterial counterpart, the cytochrome bc_1 complex, but contains cytochrome *f* instead of cytochrome c_1. The PS II utilizes manganese (Mn), calcium (Ca^{2+}) and chloride (Cl^-) ions to split water and deliver the electrons to Z, which is a tyrosine residue on the D1 polypeptide. The other PS II reaction center components are similar in function to those of the purple bacteria but involve chlorophyll, pheophytin and plastoquinone instead of their bacterial analogs. One or two molecules of bicarbonate (HCO_3^-) also appear to bind to Fe and/or near the quinones, and facilitate electron/proton transfer from Q_A to $Q_B^{(-)}$. Apparently, HCO_3^- plays no role in purple photosynthetic bacteria. PS I has a pair of primary donor chlorophyll molecules whose long-wavelength absorption maximum is at 700 nm (P700). The light-generated excited state (P700*) donates an electron to another chlorophyll molecule (A_o), and the resulting cation ($P700^+$) is re-reduced by plastocyanin. From A_o, the electron continues to move in an energetically downhill process to a phylloquinone (A_1, a vitamin K_1 molecule), and then through a series of iron-sulfur centers (FeS_x, FeS_A, FeS_B and Fd or ferredoxin). At the end of the chain, a complex known as the ferredoxin-$NADP^+$ oxidoreductase (FNR) transfers two electrons and a proton to convert $NADP^+$ to NADPH. A proton gradient created between the outside of the membrane (the stroma) and the lumen is dissipated through the coupling factor complex (CF_o — CF_1) to produce ATP.

The relative ability of a compound to accept or donate an electron can be gauged by its midpoint redox potential (E_m). These values are also shown in the diagram. A compound with a higher (more positive) E_m is more oxidizing. A compound with a lower (more negative) E_m is more reducing. Thus, in plant and cyanobacterial photosynthesis, light energy is needed to remove electrons from H_2O in the highly positive H_2O/O_2 couple and to add electrons to $NADP^+$ in the highly negative $NADP^+$/NADPH couple. Thermodynamic measurements indicate that the oxidation of two H_2O molecules into O_2, $4H^+$, and $4e^-$ should require an average oxidizing potential of about +0.81 volts for each electron removed at pH 7.0. Estimation of the Em, 7 (the midpoint redox potential at pH 7.0) of $P680/P680^+$ in the plant reaction center gives a value of about +1.1 to +1.2 volts. This value places an upper limit on the oxidizing potential available from each photon of red light absorbed by Photosystem II. The bacterial systems, in contrast, absorb longer-wavelength photons of lower energy.

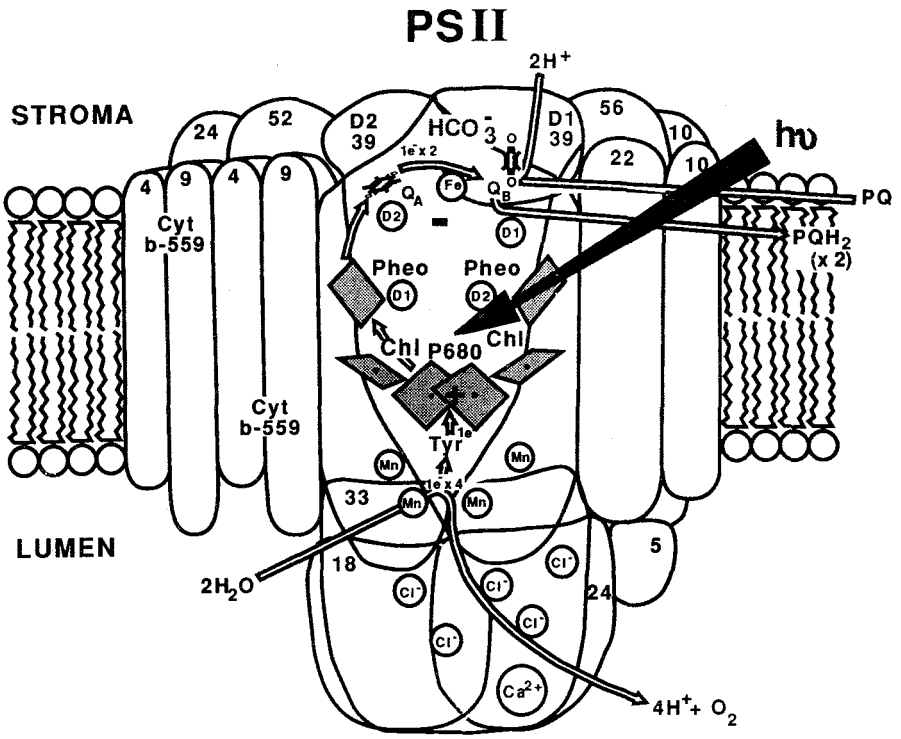

Figure 2. The photosystem II complex. The reaction center core consists of the D1 and D2 polypeptides (39 kD) which are closely associated in the membrane with cytochrome *b*559 (consisting of 4 and 9 kD subunits). The manganese responsible for catalyzing the water oxidation reactions is believed to bind to the D1 and D2 polypeptides. Numbers on the polypeptides refer to the actual or apparent molecular weights of the subunits. Polypeptides of molecular weight 52 (CP43) and 56 kD (CP47) are part of the chlorophyll-binding antenna complex. Other intrinsic polypeptides have an unknown function. Several extrinsic polypeptides (33, 24, 18 and 5 kD), forming part of the complex, appear to be involved in enhancing Ca^{2+} and Cl^- binding. The positions of the chromophores and the pathway of electron transfer within the reaction center are based largely on analogy to the purple bacterial system. In cyanobacteria, the 18 and 24 kD polypeptides on the lumen side are absent.

A major breakthrough in the field took place when the reaction center complex of the photosynthetic bacterium *Rhodopseudomonas viridis* was crystallized in 1982 (Michel, 1982). Elucidation of the three-dimensional structure by X-ray diffraction has made it possible to better understand how the light-driven charge separation is achieved (Deisenhofer and Michel, 1989). In idealized form, the reaction center mechanism involves five components: a primary donor chlorophyll pigment (P), capable of being converted to a reducing (i.e., electron-donating) excited state (P*) followed by oxidation to P^+, an electron-deficient ground state; a secondary electron donor (D) which can reduce P^+; a pheophytin electron acceptor (Pheo) which can accept an electron from P*; a primary quinone electron acceptor (Q_A), which is

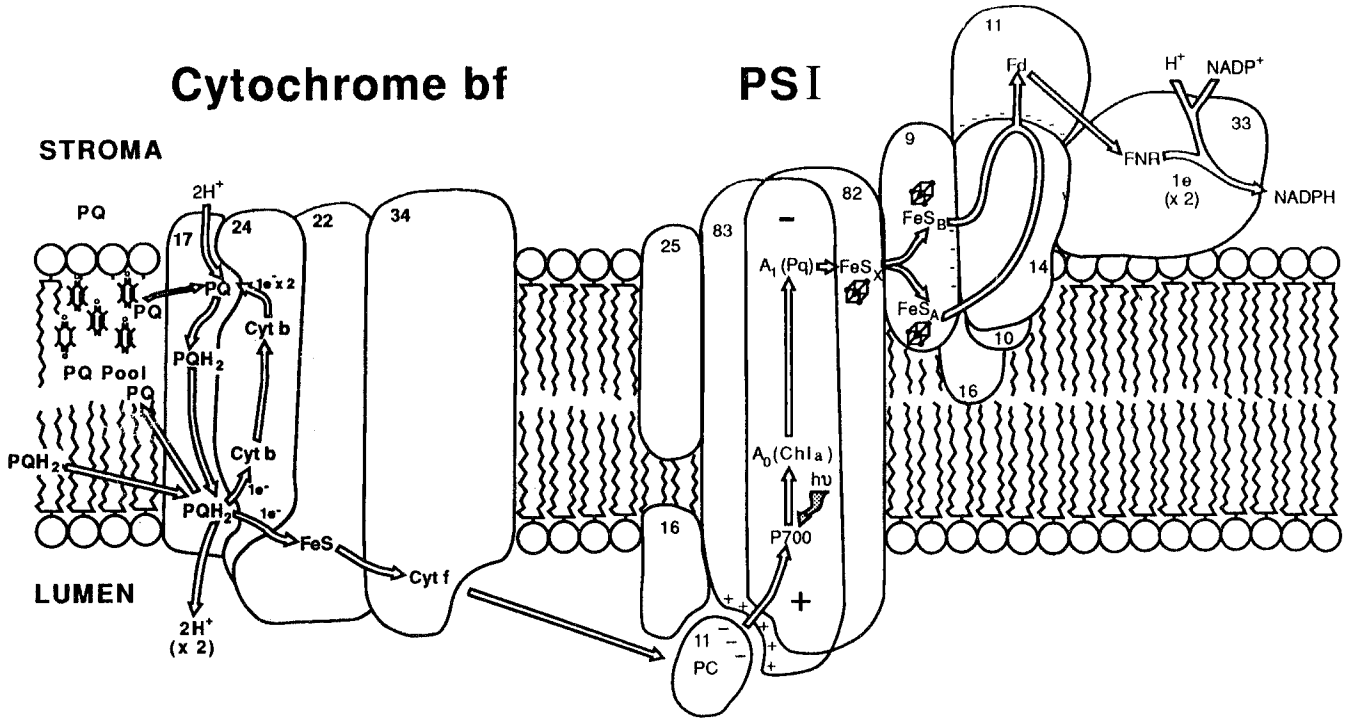

Figure 3. The cytochrome *bf* complex and photosystem I. Reactions in which plastoquinol is oxidized and plastocyanin is reduced are shown to occur in the cytochrome *bf* complex. The function of the photosystem I (PSI) complex is to oxidize the reduced plastocyanin and reduce $NADP^+$. Soluble cytochrome *c* and flavodoxin can replace plastocyanin and ferredoxin in cyanobacteria and some red algae. Numbers on the polypeptides refer to the actual or apparent molecular weights of the subunits. For other details of electron transfer, see Fig. 1 and reviews by Cramer *et al.* (1991) and Golbeck and Bryant (1991).

tightly bound to the reaction center protein; and a secondary quinone acceptor (Q_B), which is tightly bound only in its singly-reduced state, and which is otherwise capable of diffusing between protein complexes in the membrane. A non-heme iron atom (Fe^{2+}) is situated between Q_A and Q_B, but does not directly participate in electron transport in bacteria. These components are anchored in a fixed arrangement within the scaffolding of the reaction center protein complex, such that together they span the thickness of the photosynthetic membrane. Absorption of a photon of light ($h\upsilon$) by P (transforming it from P to P*) or transfer of an excition to P initiates the charge separation, as the electron moves away from its positively-charged "hole".

$$D\bullet P\bullet Pheo\bullet Q_A\bullet Q_B \xrightarrow{h\upsilon} D\bullet P^*\bullet Pheo\bullet Q_A\bullet Q_B \rightarrow$$

$$D\bullet P^+\bullet Pheo^-\bullet Q_A\bullet Q_B \longrightarrow D\bullet P^+\bullet Pheo\bullet Q_A^-\bullet Q_B \rightarrow$$

$$D^+\bullet P\bullet Pheo\bullet Q_A\bullet Q_B^-$$

Because under normal circumstances the component Q_B does not leave the complex until it has acquired two electrons (and, ultimately, two protons to form Q_BH_2), this member of the electron-accepting side of the complex must wait until the reaction center absorbs a second photon and generates another charge separation before it can diffuse to the next membrane-bound electron/proton transferring complex (known as the cytochrome *bf* complex in plants and cytochrome bc_1 in bacteria), which shuttles the protons to the inner aqueous phase of the thylakoid membrane.

In plants, the cation state of the primary donor, created as a result of the charge separation, is rapidly reduced by a donor known as "Z", which has recently been suggested or shown to consist of the phenolic group of tyrosine 161 on the D1 polypeptide (Barry and Babcock, 1987; Vermaas *et al.*, 1988; Debus *et al.*, 1988a, b; Metz *et al.*, 1989). The tyrosine radical generated by this reaction is believed to act as the acceptor for the electrons removed from water by the oxygen-evolving complex (Figs. 1 and 2).

III. Primary Photochemistry

The primary reactions of Photosystem II can be written as:

$^1Chl\mathit{a} + h\upsilon \xrightarrow{1} {}^1Chl\mathit{a}^*$ (light absorption; creation of excited state)

$^1Chl\mathit{a}^* + P680 \xrightarrow{2} {}^1Chl\mathit{a} + {}^1P680^*$ (creation of singlet excited state of P680)

$^1P680^* + Pheo \xrightarrow{3} {}^1P680^+ + Pheo^-$ (primary charge separation)

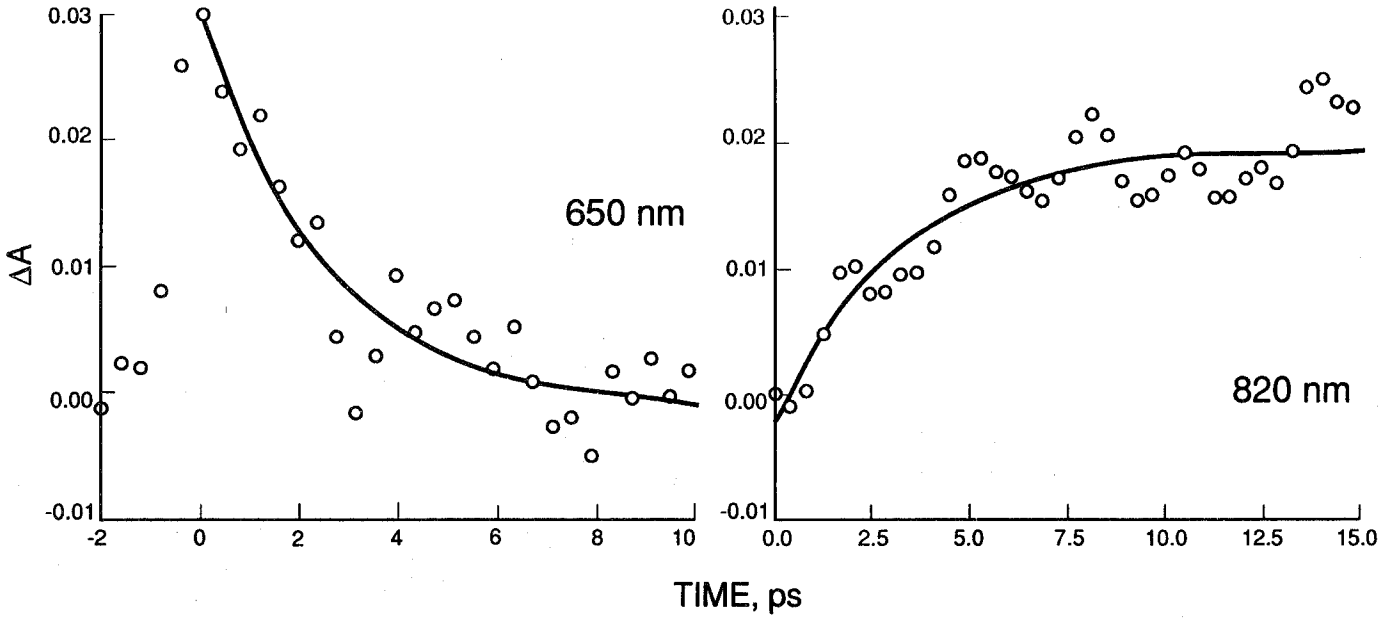

Figure 4. Primary photochemistry of photosystem II. Transient absorption changes at 650 nm (due to decay of ^{1}Chl*a**) and at 820 nm (due to formation of $P680^{+}$) for photosystem II reaction centers following a 100 μJ, 500 fs laser flash at 610 nm. Left: absorption change at 650 nm; right: absorption change at 820 nm (after Wasielewski *et al.*, 1989).

$^1P680^+ + Pheo^- \xrightarrow{4} P680 + Pheo$ (charge recombination, or loss of charges by other mechanisms)

$^1P680^+ + Pheo^- \xrightarrow{5} {}^3P680 + Pheo$ (triplet state formation)

Here, P680 is the reaction center chlorophyll *a* and pheophytin is the primary electron acceptor. For a general background on the primary events of photosynthesis, see Govindjee and Govindjee (1974).

The act of light absorption is the most rapid reaction and is estimated from the interaction of light with Chl *a* molecule. For example, Chl *a* goes into first singlet excited state by absorbing red light (680 nm). The 680 nm light oscillates with a frequency of 4.41×10^{14} cycles s^{-1}. Thus, one transition (or cycle) occurs in 2.5×10^{-15}s or 2.5 femtoseconds. This, then, is the approximate time of excitation (reaction 1). The excitation energy transfer in a reaction center must also be very rapid, considering the rise time for $^1P680^*$ production to be within the 500 fs instrument function (reaction 2). Wasielewski *et al.* (1989) have measured the lifetime of reaction 3 to be 3.0 ± 0.6 picoseconds in stable reaction center II preparations (to be discussed below). The lifetime of $P680^+$ — $Pheo^-$ (reaction 4) had been measured in reaction center II, prepared according to Nanba and Satoh (1987), to be 32 nanoseconds (ns) (see Takahashi *et al.*, 1987). Hansson *et al.* (1988) have reported that the lifetime of $P680^+$ — $Pheo^-$ depends upon the number of antenna Chl *a* remaining attached to the reaction center. Okamura *et al.* (1987) have shown the formation of the triplet state of P680, and have established the radical pair nature of its precursor through its spin polarization characteristics. Nuijs *et al.* (1986a, b) have studied primary reactions, using a 35 ps laser, in photosystem II particles enriched in reaction centers (P680/80 Chl). This study, however, does not provide direct answers to the steps involved in reaction 3 above. We discuss below the direct measurement of the kinetics of charge separation in a stable reaction center of plant photosynthesis, using 500 fs laser flashes.

Wasielewski *et al.* (1989) provided the first direct measurement of charge separation in stabilized PS II reaction centers, at 4 C, with a 500 fs resolution. Using 610 nm exciting flashes, they observed the following: (1) an instrument-limited (0.5 ± 0.4 ps) decrease in absorbance at 670 nm and 485 nm, indicating the formation of the singlet excited state of P680 ($^1P680^*$); (2) an increase in absorbance at 820 nm (due to the formation of $P680^+$) with a time constant (1/e) of 3.0 ± 0.6 ps; (3) a decrease in absorbance at 670 nm and an increase in absorbance at 485 nm or at 538 nm reflecting the formation of $P680^+$ $Pheophytin^-$ with a time constant also of approximately 3 ps; and (4) a decrease in absorbance at 650 nm (at the isosbestic point for the $P680^+$ $Pheo^-$ — P680 Pheo changes), reflecting the decay of $^1P680^*$ with a time constant of 2.6 ± 0.6 ps that matches, within experimental error, the formation of $P680^+$ (Fig. 4). Furthermore, it was shown that when Pheo was prereduced by the addition of sodium dithionite, methylviologen and light, only the absorbance changes due to the formation of $^1P680^*$ were observed; the absorbance increase at 820 nm was eliminated, confirming that the observed changes are indeed due to the charge separation.

Since the back reaction between $Pheo^-$ and $P680^+$ has been measured to be 30 ns (Takahashi *et al.*, 1987; Danielius *et al.*, 1987) in Satoh and Nanba's D1, D2, cyt *b*-559 preparations, and is expected to be 2-4 ns in intact PS II, the forward electron flow from $Pheo^-$ to Q_A is expected to be many orders of magnitude faster. Based on the measurements of the lifetime of chlorophyll *a* fluorescence of 180 to 300 ps from PS II in algae when the reaction centers are fully open, the reduction time of Q_A is suggested to be 300 ps (see Moya *et al.*, 1986; Holzwarth, 1987, for reviews on lifetime of fluorescence measurements). Our own (J. Fenton, N.S. Rao, E. Gratton. 1982, presented at the Midwest Photosynthesis Congress, Argonne, IL, USA) unpublished measurements of 200 ps in spinach thylakoids is in agreement with this conclusion. Since $P680^+$ to P680 reaction occurs in 20 – 30 ns ($P680^+$ is a quencher of Chl *a* fluorescence), the reduction time of Q_A to Q_A^- cannot be measured by fluorescence. However, the electron transfer from $Pheo^-$ to Q_A can be measured by absorbance changes. Nuijs *et al.*, (1986a), from measurements with 35 ps resolution, concluded that the electron transfer from $Pheo^-$ to Q_A occurs with a time constant of 270 ps in spinach PS II preparations enriched in P680 (1P680/80 Chl *a*). Eckert *et al.* (1988) have measured that $Pheo^-$ transfer electrons to Q_A within 200 ps. On the other hand, Schatz *et al.* (1987), using *Synechococcus* PS II preparations enriched in P680 (also 1P680/80 Chl *a*), estimated that the electron transfer from $Pheo^-$ to a subsequent quinone electron electron acceptor takes place in ~ 500 ps. Furthermore, the quantum yield of photoreduction of Q_A has been estimated to be 0.9 (see Thielen and van Gorkom, 1981). It is thus clear that no other reaction, such as the reduction of $NADP^+$ by reduced $Pheo^-$ (Arnon and Barber, 1990), can compete with the rapid and efficient reduction of Q_A by $Pheo^-$.

IV. The Oxygen Clock

Removing four electrons from water by way of four separate photoacts presents a challenging chemical problem. Although a number of basic mechanisms are possible, some are better suited than others to the energetic constraints of the system (for details, see Renger 1987a, b, 1988; Renger and Govindjee, 1985; Brudvig *et al.*, 1989). Since the chemistry is confined to a biological milieu, the toxicity of certain intermediates, such as hydrogen peroxide, may impose additional restrictions on the reaction pathway. One of the objectives in examining the catalytic turnover of the oxygen-evolving complex has been to relate the kinetics of O_2-evolution to the primary charge-separating reactions. The classical data of Emerson and Arnold (1932a, b) suggested that the photochemical reactions leading to oxygen release occur within discrete clusters of Chl known as photosynthetic units. Years later, the identification of PS II as the oxygenic photosystem (containing P680 as the primary oxidant) made it possible to study the coupling between Chl oxidation and the oxidation of water. It is known that the component $P680^+$ must eventually (by way of Z) extract an electron from water, but the rate at which each electron arrives has been found to vary in a periodic fashion. Measurements of the decay of $P680^+$ following an excitation flash indicate the oxidized primary donor recovers an electron in darkness at different rates, depending on the number of flashes given to the system (van Best and Mathis, 1978; Brettel *et al.*, 1984; Sonneveld *et al.*, 1979; Schlodder

et al., 1985). For example, the halftime for the conversion of $P680^+$ to P680 is approximately 20 ns after the 1st and 5th flashes, but is much longer after the 2nd, 3rd and 4th flashes. The periodicity of four indicates that a cyclic reaction with four steps in involved in donating electrons to the reaction center.

The relationship between the periodicity of four in electron transfer to $P680^+$ and the production of oxygen from water becomes obvious when one compares the above data with the flash number dependence of O_2 release. Using a sensitive platinum electrode, Joliot *et al.* (1969) observed that the amount of O_2 evolved after a series of short, saturating flashes oscillates with a period of four. There is no O_2 evolution after the first flash and none (or very little) after the second flash, but a maximum amount after the third flash. Thereafter, the amplitude of the O_2 yield oscillates with a period of four until the differences gradually damp out.

Kok *et al.* (1970) explained these data by proposing (see Fig. 5) that the oxygen-evolving complexes act independently of one another, and that each is capable of existing in several transient oxidation states, known as the "S states" (for different

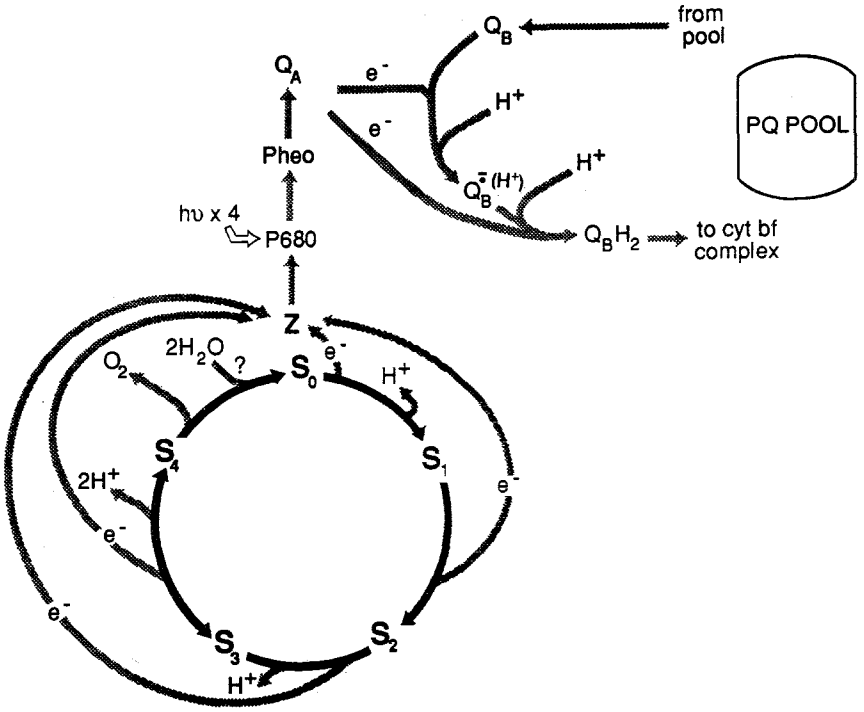

Figure 5. The four-step "oxygen clock". This mechanism delivers electrons one-by-one to the oxidized reaction center chlorophyll *a* molecule $P680^+$ as each incoming photon (hυ) oxidizes the primary donor P680. Each of the "S-state" intermediates represents an increasingly oxidized state of the oxygen-evolving complex. Protons (H^+) are shown to be released from the complex on the $S_0 \rightarrow S_1$ ($1H^+$), $S_2 \rightarrow S_3$ ($1H^+$) and $S_3 \rightarrow (S_4) \rightarrow S_0$ ($2H^+$) transitions. But, not only this release may be in non-integers, it may not even reflect the actual release from the "S" states, but from the protein. Molecular oxygen (O_2) is released only on the final transition. It is not yet known at what stage the two H_2O molecules bind or when the O—O bond is formed. On the other side of the photosystem, the two electron "quinone gate" removes reducing electrons in pairs (along with protons) for transport to the cytochrome *bf* complex. Here again, protons may first bind to the proteins.

models, see Mar and Govindjee, 1972). We call this the "oxygen clock" (see Govindjee and Coleman, 1990). The precise chemical nature of each state was, however, unknown. The need for a cycle having a periodicity of four arose from the assumption that it would take four positive charges, from four consecutive photoacts at P680, to oxidize two molecules of H_2O to yield a molecule of O_2. Advancement of each S-state during a series of flashes is limited by a dark relaxation step (i.e., an intervening S'-State), such that a finite time interval must elapse before the next incoming quantum can be utilized (Kok *et al.*, 1970; Bouges–Bocquet, 1973; Joliot and Kok, 1975). Dark relaxation steps may reflect a rate limitation on PS II turnover due to the reoxidation of Q_A^- on the $S_0' \rightarrow S_1$, $S_1' \rightarrow S_2$ and $S_2' \rightarrow S_3$ transitions. However, the $S_3' \rightarrow S_4 \rightarrow S_0$ transition appears to be limited by reactions at the water-splitting site (Joliot *et al.*, 1966; Bouges–Bocquet, 1973). A second intermediate state for the $S_2 \rightarrow S_3$ transition (S_2''), has also been proposed in order to explain the sigmoidal kinetics of this relaxation step (Bouges–Bocquet, 1973).

The other curious features of Joliot's results (the maximum on the third rather than the fourth flash and the damping-out of the oscillation with an increasing number of flashes) provided an insight into the relative oxidation sate of the "resting point" of the clock, as well as the dynamics of electron transfer between the oxygen-evolving complex and P680. Kok *et al.* (1970) explained these phenomena as follows: in darkness, there is a certain proportion of two S-states, labeled S_0 and S_1, wherein S_1 (which has one more oxidizing equivalent than S_0) outnumbers S_0 by approximately 3:1. In samples dark-adapted for hours, one starts only from S_1 (Vermaas *et al.*, 1984). The predominant reaction after the first flash oxidizes S_1 to S_2. Likewise, the second flash converts S_2 to S_3 and the third flash converts S_3 to S_4. The very short-lived S_4 state (which has accumulated four positive charges from four successive reductions of $P680^+$ to P680) releases the O_2, which is quickly detected by the platinum/Ag/AgCl electrode, used in these studies. The S_4 state must then spontaneously return to the S_0 state, so that the cycle may start again. The assumption of a long-lived S_1 state which is stable in the dark helped to explain the puzzling observation of maximal O_2 yield on the third flash. The predominance of S_1 as the resting state occurs because in darkness the S_2 and S_3 states deactivate to S_1 (on the timescale of seconds) by losing their oxidizing equivalents (Forbush *et al.*, 1971). The S_0 state is also gradually oxidized to S_1 in the dark through the slow reduction of oxidized "D", a tyrosine residue on the D2 protein (Styring and Rutherford, 1987).

The observed loss of phase coherence (i.e., damping) with increasing flash number was explained by postulating the contribution of two other factors. First, that a small probability exists for the failure of a given state S_n to advance to the S_{n+1} state (termed a "miss"; Kok *et al.*, 1970; Forbush *et al.*, 1971). This phenomenon could result from either closure of some of the phototraps (due to the presence of Q_A^-) or from a charge recombination within the system. Second, that a "double hit" occurs when the flash duration is just long enough to allow two photons to be absorbed by the same photosystem, permitting two turnovers of the S-states (Weiss and Sauer, 1970; Kok *et al.*, 1970). This explains the small amount of O_2 often observed after flash 2. When these complications are taken into account, Joliot *et al.*'s data, including

the damping of the oscillations of O_2 yield with increasing flash number, become readily understandable. After a large number of flashes, the S-states reach an equilibrium, such that the concentrations of S_0, S_1, S_2 and S_3 become equal and there is no longer any oscillation in the yield of O_2. The question of whether the value of the miss parameter (i.e., the probability) is identical or different for all of the S-state transitions has not been resolved (Delrieu, 1974; Wydrzynski, 1982).

V. The Manganese Complex as the Charge Accumulator

Discoveries of Joliot *et al.* (1969) and Kok *et al.* (1970) regarding the "oxygen clock" opened up the black box of oxygen evolution, but they still did not explain the physical make-up of the clock itself (the oxygen-evolving complex) or the chemistry of water in each of the S-states. Their experiments set off a long search for the chemical nature of the "charge accumulator" whose oxidation states are expressed by each of the numbered S-states. In the beginning, this elusive chemical entity was labeled with the letter "M", perhaps becasue it was assumed to be a metal atom (see discussion by Wydrzynski, 1982). Even before the "clock" was discovered, manganese (Mn) was suspected to comprise at least part of "M", because it has long been known that four Mn atoms per P680 are essential for O_2 evolution (e.g., see Yocum *et al.*, 1981). Current models for the location of the four Mn atoms place them on the lumenal side of the D1–D2 RC complex (e.g., see Coleman and Govindjee, 1987). Several lines of evidence suggest that two of the Mn are in an environment that is different from that of the other two (e.g., see Kambara and Govindjee, 1985). The catalytic site for water-splitting is believed to consist of a minimum of two Mn atoms (e.g., see Haddy *et al.*, 1989).

If one removes these Mn atoms, O_2 evolution is abolished, but the light-driven electron transfer from Z to $NADP^+$ remains intact, provided that artificial electron donors are supplied to the system (see Kok and Cheniae, 1966). These early studies established the role of Mn in O_2 evolution. Theoretically, Mn is a logical choice for the water-splitting catalyst, since it is capable of existing in several different oxidation states and is also known to catalyze reactions with H_2O_2 and O_2^- in other enzymes (Reed, 1986).

Many of the biologically relevant Mn complexes are paramagnetic, and a number of highly-sensitive measuring techniques have exploited this fact (Reed and Markham, 1984). Most notably, electron paramagnetic resonance (EPR) spectroscopy has been used to look at light-induced changes in the electronic structure of the Mn complex. Nuclear magnetic resonance (NMR) spectroscopy has also been used to examine the paramagnetic properties of the Mn in each observable S-state, by indirectly monitoring the ^1H_2O protons that are in contact with the metal atoms.

The question of the oxidation state of Mn has been approached from several different angles. Wydrzynski *et al.* (1975, 1978) pioneered the use of water proton (^1H) NMR to demonstrate dynamic changes in the oxidation state of Mn in chloroplast membranes. Srinivasan and Sharp (1986a, b) have unambiguously shown that changes in the S-states are related to redox changes in Mn. Tentatively, $S_0 \rightarrow S_1$ is assigned to a Mn(II) $\rightarrow$ Mn(III) transition, and $S_1 \rightarrow S_2$ to a Mn(III)$\rightarrow$Mn(IV) transition; however, no redox change in Mn is attributed to the $S_2 \rightarrow S_3$ transition.

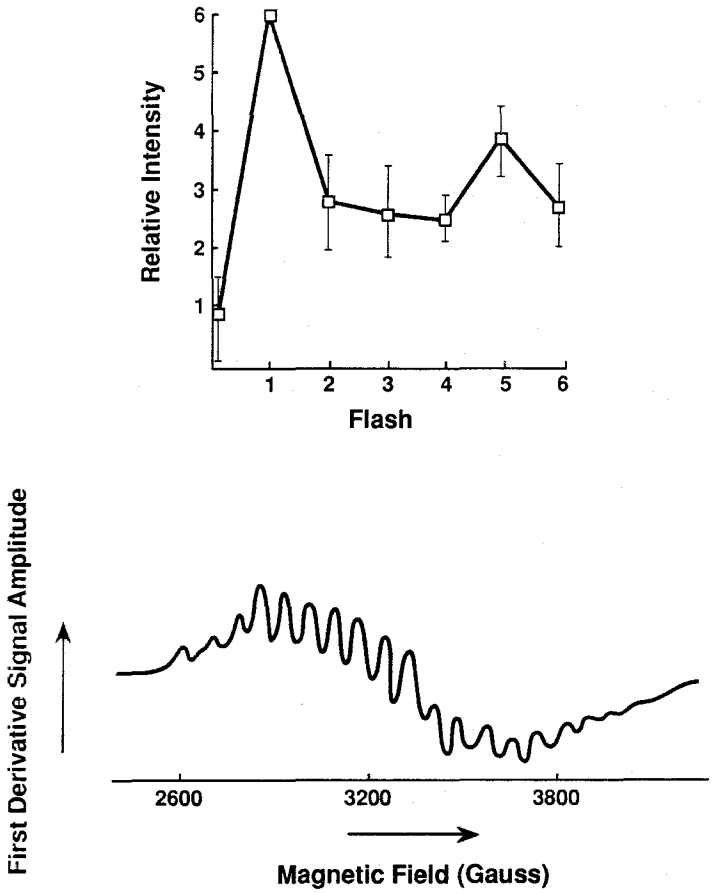

Figure 6. Electron paramagnetic resonance (EPR) spectroscopy of the oxygen clock frozen in the S_2-state. *Top* : Flash number dependence of the EPR signal; *bottom* : EPR signal at low temperature. The multiline signal is attributable to an oxidized manganese complex with unpaired electrons. The period-4 oscillation in the signal intensity versus flash number suggests that it is associated with the water-splitting reactions. Comparing the signal with those from synthetic Mn model complexes indicates that the oxygen-evolving system may contain a Mn(III) –Mn(IV) binuclear or $Mn(III)_3$ –Mn(IV) tetranuclear complex in the S_2-state (from Dismukes and Siderer, 1981).

Room-temperature EPR assays of Mn released by heating following a series of flashes have indicated a period of four oscillations in the estimated valence state of the Mn *in situ*, which suggests that the protein-bound Mn is present in both the Mn(II) and Mn(III) states, with Mn(IV) as a transient intermediate (Wydrzynski and Sauer, 1980). These results and conclusions require confirmation and extension.

Later, more direct methods were developed to observe the oxidation state of Mn by EPR at low temperature. Dismukes and Siderer (1981) reported the discovery of a 16–18 line Mn EPR signal in membranes that had been given one flash (which converts S_1 to S_2) immediately before being frozen to liquid helium temperatures. The amplitude of this signal oscillates with a period of four, indicating its involvement

in the O_2 clock, but has a maximum on the first and fifth flashes, suggesting its identity with the S_2 state (Fig. 6). The signal also contains information about the electronic structure of the S_2–Mn complex. A comparison with signals obtained from model compounds suggests that the S_2 state may arise from a mixed-valence Mn(III)–Mn(IV) dimer or a Mn(III)$_3$–Mn(IV) tetramer, although other explanations cannot be excluded (see discussions by Dismukes, 1986, 1988).

An important conclusion regarding the possible association of water with the S_2–Mn complex has emerged in a related study. Isotope substitution experiments have shown that replacing $H_2{}^{16}O$ with the ^{17}O isotope broadens the low-temperature S_2 state multiline EPR signal, which suggests that an exchangeable oxygen atom is associated with the catalytic Mn during the formation of S_2 (Hansson *et al.*, 1986). In another type of labeling experiment, $H_2{}^{16}O$ was replaced by $H_2{}^{18}O$ in samples which were poised in the S_3 state. The O_2 generated on a subsequent flash was found by mass spectroscopy to contain predominantly ^{16}O (Radmer and Ollinger, 1986). Although this experiment indicates that the bound O atoms within the catalytic site are exchangeable on the time scale of the experiment, they do not conclusively demonstrate that H_2O binds only at the level of the S_4 state. Isotope exchange experiments do indicate, however, that the Mn active site is in contact with the solvent during the S-state transitions.

An even more direct measurement of both the Mn oxidation state and the immediate physical environment of the metal has been obtained by X-ray absorption edge (XAE) and extended X-ray absorption fine structure (EXAFS) spectroscopy (see reviews by Sauer *et al.*, 1988 and Sauer *et al.*, 1991). EXAFS measurements have determined that there are several N or O ligands at a distance of about 1.9 Å from the Mn (Yachandra *et al.*, 1986a; George *et al.*, 1989). At least two Mn exist in the S_1 state as a binuclear complex, with an approximate metal-metal distance of 2.7 Å (Kirby *et al.*, 1981). This distance is compatible with a μ_2–oxo or di–μ_2–oxo bridged structure (Kirby *et al.*, 1981; Yachandra *et al.*, 1986a; George *et al.*, 1989; Guiles *et al.*, 1990a), which has been observed in some Mn model complexes (Pecoraro, 1988). Bridges composed of μ_2–hydroxo linkages are also possible. The 2.7 Å separation remains constant in the S_0, S_1, S_2 and S_3 states (Guiles *et al.*, 1987; Sauer *et al.*, 1988). Polarized EXAFS measurements indicate that the 2.7 Å vector is oriented roughly perpendicular to the membrane normal (George *et al.*, 1989). A 3.3 Å vector, oriented parallel to the membrane normal, has been attributed to a second Mn–Mn pair (see e.g., George *et al.*, 1989). However, the low intensity of this peak does not preclude the possibility that one of the scatterers is a metal atom with a lower Z value than Mn. A peak at still greater distance (~ 4.3 Å) has also been observed, and could perhaps reflect a Mn-Ca interaction (Penner–Hahn *et al.*, 1989).

Measurements of the X-ray absorption edge indicate that the energy increases when S_0 is converted to S_1 (Guiles *et al.* 1990a), and that an additional increase occurs when S_1 is converted to S_2 (Yachandra *et al.*, 1987). These results suggest that S_2 is more oxidized than S_1, and that S_0 is more reduced than S_1; however, no further change in the position of the edge is discernible after the transition from S_2 to S_3 (Goodin *et al.*, 1984; Sauer *et al.*, 1988; Guiles *et al.*, 1990b), suggesting that the oxidizing equivalent generated during this step is not stored on the Mn itself. This result raises the possibility that a redox-active component other than Mn stores

the additional oxidizing equivalent in the S_3 state. Kambara and Govindjee (1985) had discussed the possibility of a redox active ligand. One candidate for this role is a histidine radical. The idea of a manganese-histidine cluster was first discussed in detail by Padhye *et al.* (1986) and has now been adopted by others (e.g., see Rutherford, 1989). An assignment of histidine residues in the D1 protein as possible ligands for manganese has been supported both theoretically (Coleman and Govindjee, 1987; Dismukes, 1988) and experimentally (*e.g.*, see Tamura *et al.*, 1989; Preston and Seibert, 1990; Ono and Inoue, 1991). Manganese may, however, be associated with other carboxylic group containing amino acids also (Coleman and Govindjee, 1987). For example, examination of site-directed D2 mutants of *Synechocystis* sp. PCC6803 suggests that Glutamic-69 of D2 may be one potential ligand to Mn involved in O_2 evolution (Vermaas *et al.*, 1990).

A third technique that has been used to study the chemical composition of the S-states (in addition to the magnetic resonance and X-ray measurements just described) is optical spectroscopy, since manganese complexes have unique absorption bands in the ultraviolet. Several laboratories have measured flash-induced ultraviolet absorption changes that are attributed to Mn (e.g., see Dekker *et al.*, 1984a, b, c; Lavergne, 1987; Renger and Hanssum, 1988). Recent evidence suggests that the $S_0 \rightarrow S_1$ transition converts an Mn(II) ion to Mn(III), but that subsequent transitions are all Mn(III) $\rightarrow$ Mn(IV) (Kretschmann *et al.*, 1988). It is clear from all of these studies that Mn undergoes dynamic changes, including changes in its oxidation state, during the S-state transitions, although the exact electronic state of Mn corresponding to each S-state remains to be discovered. It is important to note that Boussac *et al.* (1990) have observed absorbance changes, during the S_2 to S_3 conversion in Ca-depleted membranes, that are ascribable to redox changes in histidine (see above). Finally, Lavergne (1991) has suggested that this S_2 to S_3 transition in normal membranes may involve histidine or tryptophan.

Despite the many ingenious attempts to understand the role of Mn in removing electrons from water, the electrons by themselves are not the whole story. Clearly, protons must also be accounted for as products of the water oxidation reactions. Here, however, the question arises: are all four protons released at once, simultaneous with the release of O_2, or are they liberated with each S-state transition? This question was seemingly answered by careful measurements of proton release after a series of flashes, using a highly sensitive pH electrode or pH–sensitive dyes. Fowler (1977) and Saphon and Crofts (1977) demonstrated that four protons are released sequentially from the oxygen-evolving complex: one during $S_0 \rightarrow S_1$, none during $S_1 \rightarrow S_2$, one during $S_2 \rightarrow S_3$, and two during $S_3 \rightarrow S_4 \rightarrow S_0$. After making an important correction in the earlier work, Jahns *et al.* (1991) observed that H^+ release pattern is 1, 0.5, 1 and 1.5 (instead of 1, 0, 1 and 2, as mentioned above). These and other results (e.g., see Förster and Junge, 1985; Renger *et al.*, 1987) have important implications for the mechanism of the O_2 clock, although the interpretation depends on whether the protons originate directly from water or from some other source (e.g., the polypeptides). Sequential extraction of protons from bound water implies that the substrate ligand itself (H_2O) is undergoing chemical changes prior to the S_4 state. However, if proton release from H_2O occurs in concert with O_2 release, then this implies that no water oxidation occurs until the final S-state transition. A hybrid

mechanism (stepwise proton release from water via the polypeptides) is also conceivable. It is too early to relate H^+ release pattern to water chemistry.

VI. Function of Chloride

The higher S-states accumulate some sort of net positive charge (particularly in S_2, since less H^+ release is observed during the $S_1 \rightarrow S_2$ transition). Stabilization of this positive charge may explain the essential role of chloride ions (Cl^-) in keeping the O_2 clock running. Izawa and co-workers (Izawa *et al.*, 1969; Hind *et al.*, 1969; Kelley and Izawa, 1978) demonstrated that Cl^- activates the oxygen-evolving complex. Using ^{35}Cl–NMR Critchley *et al.* (1982) and Baianu *et al.* (1984) showed that Cl^- associates and dissociates from the thylakoid membranes of halophytes freely and rapidly (with an exchange rate of greater than 3×10^4 s^{-1} at room temperature). These findings led to the proposal that Cl^- binding might be associated with the arrival of a positive charge on the oxygen-evolving complex from the oxidized reaction center $P680^+$ and that its release might coincide with the release of protons (Govindjee *et al.*, 1983). ^{35}Cl–NMR experiments by Preston and Pace (1985) suggest that Cl^- may bind more tightly in the S_2 and S_3 states than in the S_0 and S_1 states, a finding which is consistent with the more positively charged character of the higher S-states. Homann *et al.* (1986) found that when Cl^- is absent, excitation with a single flash produces an abnormal S_2 state. EPR measurements also indicate that Cl^--depleted PS II membranes form an abnormal S_2-state, whose low-temperature spectrum has a single broad peak at g = 4.1 instead of the multiline signal centered at g = 2 (Ono *et al.*, 1986). However, the g = 4.1 signal can be converted into the normal multiline signal by adding Cl^- (Ono *et al.*, 1986). Thus, Cl^- might be required for the interconversion of the S_2-state intermediate into the final relaxed state.

Despite its apparent involvement in the water oxidation reactions, direct binding of chloride to the Mn in the lower S-states was considered doubtful since EXAFS data obtained by Yachandra *et al.* (1986b) did not indicate the presence of Cl^- within 2–3 Å of the Mn atoms. Several EPR studies of the S_2-state also failed to detect Cl^- within the coordination sphere of Mn (see review by Govindjee and Homann, 1989). However, M. Klein and co-workers (personal communication) have now observed signals that they attribute to Cl^- near Mn. Homann (1987) and Coleman and Govindjee (1987) have suggested that Cl^- most likely binds to positively-charged and other specific amino acids on the oxygen-evolving complex proteins. Within the last few years, ^{35}Cl–NMR has been used to observe Cl^- binding to PS II from spinach (see Coleman *et al.*, 1987). These measurements suggest that a fairly large number of Cl^- ions bind to the oxygen-evolving complex. The bound ions appeared to be distributed between two major binding sites: one near the catalytic Mn, perhaps on the D1 and D2 polypeptides, and the other on the 33 kilodalton polypeptide. In contrast, Wydrzysnki *et al.* (1990a), also using ^{35}Cl–NMR measurements, conclude that only a single class of exchangeable chloride interaction sites occur in photosystem II.

The function of Cl^- may be to expedite the release of protons from water, and

thus increase the efficiency of the water oxidation reactions, or to stabilize the Mn ions in the higher S-states. However, it is not yet clear whether the Cl^- effects, thus far studied, are specific chemical/catalytic effects of these ions or they reflect effects of the organization of the surface properties of the proteins, i.e., conformational changes (e.g., see Wydrzynski *et al.*, 1990b). Thus, the real role of Cl^- in photosystem II remains unknown.

Coleman (1990) has summarized the role of Cl^- in *in vitro* enzyme systems. Perhaps, we should dig deeper into this problem by comparing these systems with the *in vivo* system.

VII. Role of Calcium

Calcium appears to be intimately involved in the function of Cl^-, and seems to be required both for the oxidation of water and for the operation of the PS II reaction center, perhaps in a structural or regulatory capacity. Piccioni and Mauzerall (1976) first demonstrated a potential role for Ca^{2+} in O_2 evolution in cyanobacteria. Ca^{2+} can replace the function of the 17 and 24 kilodalton polypeptides in O_2 evolution in thylakoids from higher plants (see Ghanotakis *et al.*, 1984; Ono and Inoue, 1984, Boussac *et al.*, 1985; Homann, 1988). Calcium has been shown to play an important role in controlling other proteins by acting as an intracellular messenger (switching on and off their activity) and by maintaining their three-dimensional structure (Gerday *et al.*, 1985). However, the real role of Ca^{2+} in oxygen evolution remains to be established. Removal of calcium does result in a dramatic inhibition of O_2 evolution, and this effect is reversed upon the readdition of Ca^{2+} (e.g., see Murata and Miyao, 1985). Boussac and Rutherford (1988) have shown that NaCl-induced removal of Ca^{2+} results in the inhibition of the S_3 to the S_0 step, i.e., the step in which O_2 is released. Rutherford (1989) has discussed an interesting possibility in which Ca^{2+} is located close to the Mn cluster in the oxygen-evolving complex; he imagines that Ca^{2+} acts as a shuttle in bringing H_2O and Cl^- at the right moment, i.e., at the S_4 stage, to manganese involved in water oxidation. Furthermore, Sivaraja *et al.* (1989) suggest, on the basis of citric acid induced release of Ca^{2+}, that calcium serves the purpose of a "gate keeper" for the access of substrate water to the catalytic manganese. However, as yet, no direct evidence exists that proves any direct and specific role of Ca^{2+} in the O_2 evolution process. For reviews, see Homann (1990) and Yocum (1991).

In conclusion, although a great deal of fog has been lifted from water oxidation being a mysterious reaction, yet its mechanism has only begun to be understood at the molecular level. Although manganese clearly undergoes redox changes, and histidine appears to be involved at one of the steps, yet the roles of calcium and chloride are obscure. New methods need to be invented to monitor the intermediates of water oxidation. Also, there is much need to improve methods to prepare the simplest, yet stable, reaction center complexes that will retain not only quinones, but also manganese. Such simple preparations will help us obtain meaningful data on the molecular mechanism of O_2 evolution.

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