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MINI-REVIEW

The Relations Between the Chloride, Calcium, and Polypeptide Requirements of Photosynthetic Water Oxidation

Peter H. Homann[1]

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Introduction

In biological systems, many essential functions are assumed by inorganic molecules and ions. Thus, there is nothing unusual about the requirement of at least three different inorganic cofactors in the mechanism of photosynthetic water oxidation by green plants, algae, and cyanobacteria. One of these is Mn of which each water-oxidizing site contains four, presumably protein-bound and in an elevated oxidation state. The other two are Cl^- and Ca^{2+} ions. The puzzling relations of these two ions to the extrinsic polypeptides at the water-oxidizing site is the subject of this contribution.

The surge of research activity dealing with various aspects of the water-oxidizing photosystem II (PSII) has led to the almost simultaneous appearance in the literature of several review articles (Critchley, 1985; Renger and Govindjee, 1985; van Gorkom, 1985; Govindjee *et al.*, 1985; Ghanotakis and Yocum, 1985; Dismukes, 1986). For a detailed discussion of this contribution's background, the reader may wish to consult these reviews, and the other articles of this series.

The Organization of the Water-Oxidizing Site

Photosynthetic reduction of $NADP^+$ is accomplished at the expense of an oxidation of water in PSII. Molecular oxygen is the final oxidation

[1]Institute of Molecular Biophysics and Department of Biological Science, Florida State University, Tallahassee, Florida 32306-3015.

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product. It is formed by abstraction of two electrons from each of two water molecules according to

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

The necessary four oxidizing equivalents are accumulated by successive light reactions, generating the oxidation states S_1, S_2, S_3, and S_4 of the water-splitting enzyme. S_4 is assumed to spontaneously produce O_2 from water as it converts to S_0. No evidence exists for the intermediate formation of free partially oxidized derivaties of water, but two of the four H^+ are released in steps preceding the liberation of O_2.

The electron carriers between water and the reaction center P-680 usually are divided between the water-oxidizing S complex and the reaction center complex or trap. The latter encompasses P-680, its primary electron acceptors pheophytin, the plastoquinones Q_A and Q_B, as well as the primary donors Z and, presumably in a side path, D, both being suspected to be plastoquinols. The function of another constituent, cytochrome *b*-559, is unknown. "Disconnections" of the two complexes are, in general, difficult to localize because of the existence of perhaps an additional intermediate electron carrier.

With regard to the functional entity responsible for water oxidation, there is general agreement that at least two of the aforementioned four Mn represent the charge accumulating "S" complex at the active site of "water oxidase." Some Mn may be bound to an intrinsic polypeptide with a molecular

Table I. Some Polypeptides in and around the Water-Oxidizing Complex[a]

Molecular mass		Membrane association	Tentatively assigned role
Spinacia	*Synechococcus*		
47	47	Intrinsic	P-680 or chl *a* (?) binding
43	40	Intrinsic	chl *a* antenna
34	30	Intrinsic	Mn-binding? D_2?
32	30	Intrinsic	Q_B (herbicide) binding
22[b]	?	Intrinsic	23-kDa binding
9	9	Intrinsic	? (cyt-*b*559)
33	34	Extrinsic	? ("Mn-stabilizing")
23	–	Extrinsic	? ("Cl-retaining")
17	–	Extrinsic	? ("H^+ conducting"?)
10	–	Extrinsic	?
–	(9)[c]	Extrinsic	"Regulatory"
5	–	Extrinsic?	?

[a]Molecular mass as determined by SDS gel electrophoresis. For *Spinacia* data see review by Ghanotakis and Yocum (1985). *Synechococcus* data from Yamagishi and Katoh (1984), Satoh *et al.* (1985), and Koike and Inoue (1985); these authors, like Stewart *et al.* (1985) for *Phormidium*, did not detect any of the extrinsic polypeptides other than the 34-kDa species.
[b]From Ljungberg *et al.* (1984).
[c]Data for *Phormidium* from Stewart *et al.* (1985).

mass of approximately 34 kDa, and the quinones probably are associated with smaller polypeptides of masses in the 30–34 kDa range.

Four extrinsic polypeptides have been assigned to the domain containing the water-oxidizing complex. The molecular masses of the polypeptides isolated from spinach (*Spinacia oleracea*) thylakoids are approximately 33, 23, 17, and 10 kDa, and the abundance of the first three has been estimated to be 1 or 2 per reaction center. Since not much is known about the smallest one, it will be neglected in this article.

Table I lists known polypeptides of PSII together with a brief note about possible or established functions as far as known. For comparison, the corresponding polypeptides from cyanobacterial thylakoids are also given, but the assignment of analogy must, in several cases, be considered tentative due to the scarcity of relevant data. This deplorable situation explains the rather cursory treatment of the cyanobacterial PSII in the following discussions.

Creating Cl^- and Ca^{2+} Deficiency at the Water-Oxidizing Site

The essential role of Cl^- and Ca^{2+} in oxygenic electron transport was not, as in the case of Mn, discovered in analyses of nutritional requirements of living organisms. The obvious reason is the multitude of other essential functions these ions have to fulfill. It is not surprising, therefore, that some of the nutritional studies that were made with Cl^- (Grimme and Kessler, 1970; Terry, 1977) have not helped to clarify the issue. It has been learned, however, that chloroplasts secure their supply of Cl^- by a well-regulated, and exceptionally efficient, Cl^- concentrating mechanism (Robinson and Downton, 1984).

The requirement for Cl^- of the photosynthetic electron transport chain was discovered by Warburg and Lüttgens (1944) more than 40 years ago. These authors also recognized that a few other monovalent anions like Br^- and NO_3^- could be substituted for Cl^-, albeit with somewhat lower effectiveness. Twenty-five years later, Hind and his coworkers (Heath and Hind, 1969; Izawa *et al.*, 1969) demonstrated that the need for activating anions was restricted to the water-oxidizing site of PSII, but this work had surprisingly little impact at the time.

The long neglect of Cl^- by researchers in photosynthesis was undoubtably due to the difficulty to reproducibly create a condition of "Cl^--deficiency," i.e., an impairment of the electron transport chain that could be remedied by an addition of Cl^-. With intact thylakoids, the problem turned out to be that a Cl^--releasing exposure of the water-oxidizing site to alkaline conditions required a pH equilibration between the external medium and the thylakoid lumen (Theg and Homann, 1982). Now that purified PSII complexes can be studied in inside-out thylakoid vesicles (Andersson and Åkerlund, 1978) or

in detergent-disrupted membranes (Berthold *et al.*, 1981; Kuwabara and Murata, 1982; Yamamoto *et al.*, 1982), a manipulation of the pH at the water oxidase is no longer a problem.

Cl^- deficiency symptoms can also be induced by an incubation of the PSII preparation in media containing moderately high concentrations of anions like SO_4^{2-} (Sandusky *et al.*, 1983) or F^- (Critchley *et al.*, 1982) which themselves are unable to support water-oxidizing activity. They, like the OH^- anions of alkaline media, are often assumed to displace Cl^- from its binding site (Critchley *et al.*, 1982; Sandusky *et al.*, 1983; Casey and Sauer, 1984; Damoder *et al.*, 1986; Sandusky and Yocum, 1986). Na_2SO_4 most effectively induces Cl^- deficiency at a slightly elevated pH (Sandusky *et al.*, 1983), but this limitation can be overcome by adding sulfates of divalent cations like Mg^{2+} (Franzén *et al.*, 1985).

As Itoh and Iwaki (1986) have pointed out, the Cl^--removing ability of both anionic and cationic solutes must reflect an involvement of electrostatic forces in the association of Cl^- with the water-oxidizing site. Homann (1986, 1987) has observed that, in the absence of Cl^-, the 23-kDa polypeptide became detached from PSII membranes of tobacco (*Nicotiana tabacum*) at pH > 6.5, especially in the presence of sulfate and divalent cations. Cl^- protected against this polypeptide loss, and a subsequent addition of this anion partially reversed the polypeptide dissociation. Hence, the electrostatic forces weakened by an elevated pH, or by divalent cations or anions, may be those responsible for the binding of the 23-kDa polypeptide which is a major barrier to both Cl^- removal (Critchley *et al.*, 1984) and rebinding (Homann and Inoue, 1985).

The experimental conditions producing a demonstrable Ca^{2+} requirement are still rather poorly defined. An early report on a Ca^{2+} involvement in PSII electron transport of cyanobacteria (Piccioni and Mauzerall, 1976) generated little attention, presumably because it was attributed to some peculiarity of the prokaryotic system. Indeed, even today it is difficult to reconcile some of the experimental data obtained with cyanobacterial membranes with those from chloroplasts. In the former, one action of Ca^{2+} appears to reside close to the reaction center P-680 and can be mimicked at least partially by Mg^{2+} or even Na^+ (Becker and Brand, 1985; Satoh and Katoh, 1985a). In contrast, the effects of Ca^{2+} at the water-oxidizing site are highly specific for this ion (Ghanotakis *et al.*, 1984a; Miyao and Murata, 1984).

A Ca^{2+}-requiring state of PSII in intact isolated chloroplasts has been reported for wheat (*Triticum aestivum*) grown under an intermittent light regime (Ono and Inoue, 1983). Such photosynthetically incompetent developing chloroplasts required Ca^{2+} for the incorporation and functionalization of Mn. These results were corroborated by *in vitro* photoreactivation experiments with thylakoid preparations of cyanobacteria (Pistorius and Schmid, 1984;

Satoh and Katoh, 1985b), and with intact chloroplast thylakoids which required Cl^- as well (Yamashita and Ashizawa, 1985).

In a fully assembled PSII, Ca^{2+} is bound so tightly that a requirement is not noted until the PSII preparation has been freed of its 23-kDa polypeptide (Ghanotakis *et al.*, 1984a; Boussac *et al.*, 1985b; Ghanotakis *et al.*, 1985a) or after treatment with trypsin (Voelker *et al.*, 1985). Yet, even then the extent of activity loss recoverable by a Ca^{2+} addition may vary considerably. For example, Ca^{2+} addition caused only a doubling of the rate of O_2 evolution with highly purified PSII membranes lacking all extrinsic polypeptides except the 33-kDa species (Ikeuchi and Inoue, 1986). Large "Ca effects" can be induced by extraction of PSII membranes with low concentrations of the chelator EGTA (Ghanotakis *et al.*, 1984a), or when the incubation in 1.5 M NaCl to effect the polypeptide depletion is performed in ambient light, and the presence of EGTA during the assay ensures sequestration of traces of contaminating Ca^{2+} (Boussac *et al.*, 1985a). Without prior Ca^{2+} removal, a requirement for this cation may develop during the assay of light-driven electron transport and become apparent as a steady activity decline (Nakatani, 1984a) and increased life time of Z^+ (Dekker *et al.* 1984). Because of irreversible photoinactivations, this decline can be reversed only partially by a subsequent Ca^{2+} addition.

Cl^-, Ca^{2+}, and the Extrinsic 33-, 23-, and 17-kDa Polypeptides

Much of what we know about the requirements of the mechanism of photosynthetic oxygen evolution has been gathered by selectively removing and reinserting components of the water-oxidizing apparatus. A minimal requirement, however, is not easily defined because not all experimentally created active systems have physiological relevance. This aspect, on the other hand, has provided rather unique sets of criteria for an assessment of the function of various inorganic and organic constitutents.

One might expect that a hierarchical organization would become evident from the sequence of events leading to the assembly of the water-oxidizing complex *in vivo*. According to Ryrie *et al.* (1984), the 33- and 23-kDa polypeptides appear at a very early stage in developing barley (*Hordeum sativum*) chloroplasts. Only the accumulation of the smaller 16-kDa polypeptide (equivalent to the 17-kDa species of *Spinacia*) correlated with the emergence of oxygen-evolving activity. However, these results are not very informative because the actual assembly of the polypeptides into the membrane may be contingent upon the full complement of functional Mn (Becker *et al.*, 1985; Kuwabara *et al.*, 1985; Ono *et al.*, 1986a). Conversely, mutations affecting these polypeptides (Cammarata *et al.*, 1984),

or even the herbicide binding protein (Andersson *et al.*, 1985; Metz *et al.*, 1985), apparently interfere with the normal assembly of a functional Mn-center.

In contrast to the message from the *in vivo* data, many *in vitro* experiments on the water-oxidizing complex suggest that the small 17-kDa polypeptide is dispensible. Some reports ascribe to this polypeptide an essential contribution to normal Cl^- relations of PSII (e.g., Izawa, 1984; Imaoka *et al.*, 1984). Whether justified or not, at this stage of our knowledge the role of the 17-kDa polypeptide has to be treated with some indifference, and subordinated to that of the 23-kDa species. This is made easier by the fact that any treatment that causes detachment of the latter also releases the former. If the 17-kDa polypeptide should remain associated with the membrane preparation, it is quite likely due to its tendency to bind nonspecifically (Miyao and Murata, 1983; Ikeuchi and Inoue, 1986).

After removal of the two small polypeptides, PSII membranes no longer retain high activities of water oxidation in Cl^--free media even at pH < 7. Instead, the activity becomes dependent on the provision of unphysiologically high concentrations of Cl^- in the assay medium (Critchley *et al.*, 1984; Imaoka *et al.*, 1984) and often of Ca^{2+} as well (see above). Comparative evaluations of published activity analyses of polypeptide-stripped PSII membranes (Åkerlund *et al.*, 1984a, b; Dekker *et al.*, 1984; Boska *et al.*, 1985; Homann, 1985; Radmer *et al.*, 1986; Tamura *et al.*, 1986) are hampered by a nonuniform Cl^- and Ca^{2+} status of the preparations, and a certain degree of irreversibility of the inflicted lesions. In spite of such uncertainties, however, the emerging consensus is that, if sufficient Cl^- and Ca^{2+} are provided, the absence of the 23-kDa polypeptide does not disrupt electron flow between the S complex and Z. Yet, it remains to be explored whether the removal of the 23-kDa polypeptide from inside-out vesicles has farther reaching consequences (Åkerlund *et al.*, 1984a, b) than its detachment from PSII particles prepared by detergent action.

The requirement of almost ten times lower Cl^- concentrations for optimal activity after rebinding of the 23-kDa polypeptide has been attributed to its "Cl^- concentrator" function (Murata and Miyao, 1985). This would imply creation of an activity gradient of Cl^- between the putative membrane pocket harboring the water oxidase (Homann *et al.*, 1983) and the external medium. The structural and energetic ramifications of such Cl^- accumulation, however, have not been adequately addressed.

A more plausible explanation is that the 23-kDa polypeptide in some way regulates the Cl^- affinity of its putative binding site (Critchley, 1985; Homann and Inoue, 1985), or that the polypeptide satisfies some role that, in its absence, can be taken over by Cl^-. The multiplicity of Cl^- action invoked by the latter concept has some experimental support. However, aside

from the nonphysiological nature of any substitution of the 23-kDa polypeptide by Cl^-, it is not perfect functionally. For example, even high concentrations of Cl^- are unable to overcome the labilization of functional Ca^{2+} (Nakatani, 1984a) or to restore full protection of the Mn from reduction and subsequent release by the action of added reductants (Ghanotakis *et al.*, 1984b; Tamura *et al.*, 1986).

The influence of the 23-kDa polypeptide on Ca^{2+} binding is controversial also. Ghanotakis *et al.* (1984a) have shown that PSII membranes which lack the 23-kDa polypeptide and require Ca^{2+} for optimal activity have to be provided with millimolar concentrations of Ca^{2+} when the polypeptide is withheld, but need only micromolar concentrations after the rebinding of the polypeptide. Data obtained by Boussac *et al.* (1985b) and our investigations (Homann, unpublished), on the other hand, have revealed a high affinity interaction regardless of the presence of the 23-kDa polypeptide. Similarly, Ikeuchi and Inoue (1986) report that the Ca^{2+} requirement of their highly purified PSII preparation is satisfied by submillimolar concentrations in spite of an absence of the 23-kDa polypeptide.

The 33-kDa extrinsic polypeptide can be removed from PSII membranes only by rather drastic procedures (see review by Ghanotakis and Yocum, 1985) which usually yield preparations lacking the other two polypeptides as well. Stripped of all three polypeptides, PSII membranes no longer have water-oxidizing activity. This can be traced to a loss of paramagnetic coupling between two of the four bound in Mn (Mavankal *et al.*, 1986) which, at room temperature, gradually dissociate from their binding sites (Ono and Inoue, 1984; Imaoka *et al.*, 1986). High concentration of NaCl ($\geqslant 100$ mM) caused recoupling and stabilization of the Mn pair (Mavankal *et al.*, 1986) and, as a consequence restored some, albeit flawed, water-oxidizing activity (Kuwabara *et al.*, 1985; Ono and Inoue, 1986). Since much lower concentrations of $CaCl_2$ reactivated the water-oxidizing mechanism to a similar extent (Kuwabara *et al.*, 1985; Franzén *et al.*, 1985), the action of NaCl cannot, however, be attributed entirely to Cl^-.

Prior to the studies just cited, many experiments with PSII preparations lacking the 33-kDa polypeptide had been performed in media containing less than 50 mM NaCl. In each case, electron transfer from the water oxidizing system was found to be impeded. EPR measurements (Franzén *et al.*, 1985; Boska *et al.*, 1985; Ghanotakis *et al.*, 1985b; Imaoka *et al.*, 1986) failed to reveal any significant S_2 formation, and the reduction kinetics of the primary PSII donor Z^+ indicated a preferential re-reduction by a backreaction with Q_A^-, and by an unidentified reductant (Boska *et al.*, 1985). Furthermore, using an artificial electron donor as probe, Tamura *et al.* (1986) detected only very little S_2. Yet, as long as no Mn was lost, Z^+ appeared to remain coupled to the Mn center(s) (Ghanotakis *et al.*, 1985b), and the oscillations

of thermoluminescence proceeded almost normally after the first two flashes (Ono and Inoue, 1985), suggesting a progress of charge accumulation to an S_3 state. As will be discussed later, such seemingly contradictory results have been obtained also under other conditions.

"Binding Sites" for Cl^- and Ca^{2+}

The dependence of the water-oxidizing activity of Cl^-- or Ca^{2+}-depleted thylakoid preparations on the concentration of the re-added ion has invited its description by Michaelis–Menten kinetics for the action of an essential enzyme activator (Kelley and Izawa, 1978; Ghanotakis *et al.*, 1985b). Such approach was also applicable when Cl^- binding was measured as broadening of the ^{35}Cl NMR linewidth (Baianu *et al.*, 1984). When the calculated K_m is used as a measure of the dissociation constant of the ion, one has to postulate that rapid equilibrium kinetics are applicable, and any redissociation of the ion after enzyme turnover is not rate limiting. An inherent presupposition of the Michaelis–Menten approach is that activation occurs by binding of one ion per enzyme. However, actual determinations of the number of ions associated with each water-oxidizing site yielded values between 4 and 40 for Cl^- (Theg and Homann, 1982; Izawa, 1984), and 2 for Ca^{2+} (Tamura and Cheniae, 1985). If true, certain assumptions must be invoked to justify the kinetic analyses (Homann, 1985) unless the kinetic data reflect among multiple binding of the ions only the rate-determining one.

Regardless of the mechanistic limitations, the kinetic analyses have helped to quantitatively describe the Cl^- and Ca^{2+} requirements of the water oxidase. They allowed, formally at least, to interpret the prevention by Cl^- of the inhibitory action of amines as a competitive interplay (Sandusky and Yocum, 1984, 1986). Since the amines might inhibit by binding directly to Mn (Velthuys, 1975), Sandusky and Yocum (1984) concluded that Cl^- was also bound there, perhaps as an inner-sphere bridging ligand serving to transfer electrons in the Mn cluster (cf. Critchley, 1985). While no other experimental evidence exists for such inner-sphere ligation of Cl^- to Mn (Dismukes, 1986; Yachandra *et al.*, 1986), light has been reported to make Cl^- more resistant to extraction (Sandusky *et al.*, 1983), and Preston and Pace (1985) have interpreted their ^{35}Cl NMR data as indicating a preferential Cl^- binding in the S_2 and S_3 states. However, ^{35}Cl NMR identifies only Cl^- that is rapidly exchanging with the bulk Cl^-, and not Cl^- in a relatively inaccessible crevice (Falke *et al.*, 1984). Thus, the results may merely prove an S-state dependence of the mode of Cl^- binding, and do not support the concept of a tight ligation of Cl^- to Mn. Moreover, both the NMR experiments and the studies of amine action have been done with preparations devoid of the 23-kDa

polypeptide, either by design (Preston and Pace, 1985) or as an unavoidable consequence of the experimental conditions (Homann, 1986). While apparently not changing the steric parameters around the active site of the water oxidase (Tamura and Cheniae, 1985), removal of the polypeptide may still allow a more intimate interaction with added Lewis bases like anions or amines. Evidence for this contention comes from my observation that the otherwise activating I^- is turned into an electron donor after removal of the 23-kDa polypeptide (Homann, unpublished).

Results from kinetic analyses of the pH dependence of Cl^- binding have revealed that the Cl^- affinity of Cl^--depleted PSII membrane particles decreases progressively down to pH 5, and presumably beyond (Itoh and Uwano, 1986; Homann, unpublished). Indeed, at pH 5 the apparent K_m for Cl^- binding was found to be as low as 20 μM for preparations from *Phytolacca americana* bearing their 23-kDa polypeptides, and approximately 300 μM after its removal. Thus, at a sufficiently low pH, the Cl^- requirement was in the physiological range regardless of the presence of the "Cl^--concentrating" 23-kDa extrinsic polypeptides (Homann, unpublished).

Unfortunately, our knowledge about anion binding in proteinaceous systems is scant. For PSII, a Cl^- association with protonated amine residues was indicated by its ability to protect photosynthetic water oxidation from amine-reactive reagents (Carpentier and Nakatani, 1985; Johnson, 1987). However, the protonation equilibrium of such groups cannot be invoked to explain the pH dependence of the observed Cl^- affinity. In analogy to the situation encountered with respect to anion adsorption to minerals (Hingston, 1981), one may attribute the observed $pk_a < 5$ instead to negatively charged entities (carboxylates) which exert control over the Cl^- association due to their proximity to the Cl^- binding site(s) (cf. Govindjee *et al.*, 1985).

Due to its manifold function in biological systems, much more is known about the interaction of Ca^{2+} with biological molecules, than about Cl^-. The preference of Ca^{2+} for oxygen ligands makes carboxyl, carbonyl, and hydroxyl groups on proteins likely binding sites. Because of similar properties, lanthanide ions can be used as Ca^{2+} probes. In thylakoids, Ca^{2+} was found to competitively prevent the inhibitory actions of lanthanide ions, but it did not reverse them, presumably because of stronger binding of the latter and a slow, lanthanide-induced dissociation of the three extrinsic polypeptides from the water-oxidizing site (Ghanotakis *et al.*, 1985b). These polypeptides are rich in aspartic and glutamic acids (see review by Ghanotakis and Yocum, 1985) and would be well suited for Ca^{2+} binding, as would be anionic lipids. However, no evidence is available for a direct binding of Ca^{2+} to any of these polypeptides. In fact, the reported persistence of a Ca^{2+} effect after removal of all three polypeptides (Franzén *et al.*, 1985; Kuwabara *et al.*, 1985) suggests that at least one of the possibly two (Tamura and Cheniae, 1985)

sites of interaction is on an intrinsic polypeptide, or a lipid, close to the water-oxidizing site. Once all polypeptides are fully sequenced, it will be possible to decide whether the unique loop region can be formed which has been implemented in Ca^{2+} binding by other proteins, including calmodulin (Reid, 1985). The first hint that this may be so comes from the work of several laboratories (Barr *et al.*, 1982; England and Evans, 1983; Pistorius, 1983; Carpentier and Nakatani, 1985) which showed that organic compounds known to be Ca^{2+} antagonists in calmodulin inhibit the electron transport close to the water oxidase, and prevent various Ca^{2+}-mediated effects in PSII. Furthermore, Sparrow and England (1984) claim to have isolated from thylakoids a Ca^{2+}-binding polypeptide using an affinity column prepared with one of those antagonists.

The Consequences of Cl^- and Ca^{2+} Deficiency for the Function of the O_2-Evolving Complex

As is the case after polypeptide removal, irreversible perturbations of the water-oxidizing site as a result of the depletion treatments are a major concern also when evaluating the consequences of Cl^- and Ca^{2+} removal. Evidence for structural changes following Ca^{2+} removal has been obtained recently (Homann, unpublished). It is not certain, furthermore, whether Cl^- depletion by Na_2SO_4, NaF, or high pH yields entirely comparable preparations. One only needs to remember that Ca^{2+} and F^- might combine to form the rather insoluble CaF_2. Another obstacle is the considerable susceptibility of a Cl^-- and/or Ca^{2+}-depleted PSII to irreversible photoinactivation which for the case of Cl^- deficiency has been localized at a site close to P-680 (Theg *et al.*, 1986).

The available data on the effects of Ca^{2+} deficiency, and of a depletion of the 17-, 23-, and 33-kDa polypeptides, have identified as the most sensitive electron transfer step the decisive one, namely the creation of the water-oxidizing S_4 state. Indications of oxidant accumulation up to an "S_3" state, or even a Z^+S_3 condition, have come from the flash-induced patterns of thermoluminescence (Ono and Inoue, 1985; Vass *et al.*, 1987), and of ms luminescence for the minus Ca^{2+} condition (Boussac *et al.*, 1985a). Yet, it was mentioned earlier that EPR and absorption spectroscopic approaches to verify the formation of a normal S_2 state have failed for samples depleted of the three polypeptides. Analyses of the thermoluminescence have revealed, in fact, that the generated "S_2" condition had unique properties (Vass *et al.*, 1987) which might be attributable to an abnormal binding and/or ligation of the manganese (cf. Mavankal *et al.*, 1986).

The consequences of Cl^- depletion have recently been analyzed in some more detail. Following the discovery of unusually stable oxidants in a Cl^--deficient PSII by Muallem *et al.* (1981), several groups of investigators have confirmed a defective oxidant storage using a variety of experimental approaches (Itoh *et al.*, 1984; Theg *et al.*, 1984; Sinclair, 1984; Homann *et al.*, 1986; Ono *et al.*, 1986b, c; Vass *et al.*, 1987). The combined data suggest that illumination of dark-adapted Cl^--depleted PSII preparations with a single flash generates an abnormal S_2 state characterized by a long lifetime and a lower than normal oxidation potential. This "Σ_2-state" (Homann *et al.*, 1986) does not display the normally seen multiline EPR signal (Damoder *et al.*, 1986; Imaoka *et al.*, 1986) while behaving like the regular S_2 state with respect to the weaker $g = 4.1$ signal (Casey and

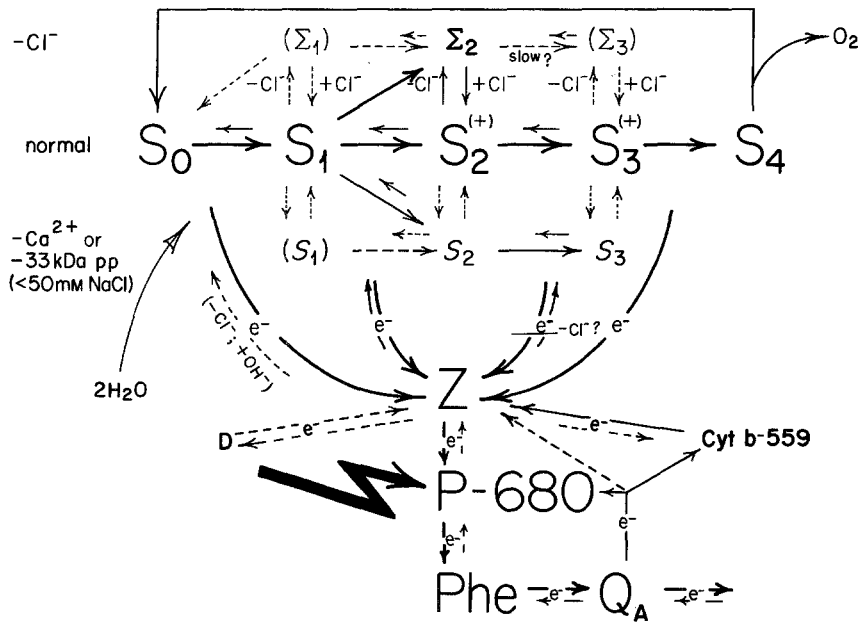

Fig. 1. Electron transfer pathways in photosystem II, and associated reactions. See text for underlying observations, concepts, and hypotheses. When the existence of intermediates is not or poorly supported by any experimental evidence, they are enclosed in parentheses (the possibility of an e-carrier between Z and S has been ignored but may explain the existence of the pseudo-S-states "Σ" and "*S*"). Broken arrows indicate purely hypothetical electron transfer paths, while solid arrows describe paths which are either confirmed experimentally, or which are postulated on the basis of some type of experimental evidence. Because the effects of the various modifications on the proton release pattern during water oxidation have not been explored, deprotonation events are not shown. The (+) at S_2 and S_3 indicates, however, that here electron removal is thought not to be compensated for by H^+ release (see review by Dismukes, 1986). However, according to Radmer and Ollinger (1986), tight binding of substrate water may not occur until the S_3 state is formed (but see Hansson *et al.*, 1986).

Sauer, 1984; Ono *et al.*, 1986c). Furthermore, the abnormal Σ_2 state and the normal S_2 state have proven to be interconvertible simply by changing the Cl^- status of the water-oxidizing complex through additions of either Cl^- or relatively high concentrations of SO_4^{2-} (Homann *et al.*, 1986; Ono *et al.*, 1986c).

The fate of the oxidant generated by a second flash is not certain. While thermoluminesence measurements provided evidence for an advance of the Σ_2 state to an analogous equally stable "Σ_3" condition, recent EPR studies by Ono *et al.* (1986c) confirmed the conclusion of Itoh *et al.* (1984) and Theg *et al.* (1984), from fluorescence analyses, that only a $\Sigma_2 Z^+$ can be formed which for yet obscure reasons could not be converted to S_3 upon Cl^- addition.

The scheme of Fig. 1 is an attempt to describe the electron flow in PSII after Cl^- depletion, as well as after dissociation of the three extrinsic polypeptides which appears to cause effects quite similar to Ca^{2+} removal (Dekker *et al.*, 1984) and may actually be equivalent to it in some respects.

Cl^- and Ca^{2+} Functions in the Water-Oxidizing Protein Complex

As contributors to the microenvironment surrounding the S-complex, Cl^- and Ca^{2+} together with the extrinsic polypeptides of masses 33, 23, and 17 kDa may be essential for a successful functionalization of Mn during "photoactivation" of the water oxidase of chloroplasts *in vivo*. The 23-kDa polypeptide ensures an environment, perhaps even ligands, which prevent the approach to the functional Mn of external reductants while allowing an efficient redox interaction with the natural oxidants. Presumably by virtue of its surface charge distribution, the 23-kDa polypeptide controls structures responsible for Ca^{2+} and Cl^- retention near the S complex. Conversely, Cl^- influences the binding of the 23-kDa polypeptide.

Before speculating about the action of Cl^- in photosynthetic water oxidation, it is helpful to list some aspects of the "Cl effect" for which there are precedents in other anion-sensitive systems: (1) the anion-induced shift of the pH optimum of the water oxidase which suggests that Cl^- addition is equivalent to an acidification (α-amylase: Urata, 1957; angiotensin-converting enzyme: Bünning and Riordan, 1983); (2) the selectivity for monovalent anions in an order closely resembling the lyotropic Hofmeister series (acetoacetate decarboxylase: Fridovich, 1963; α-amylase: Levitzki and Steer, 1974; carbonic anhydrase: Lindskog, 1983); (3) an electrostatic association of Cl^- with a protein (carbonic anhydrase: Lindskog, 1983, halorhodopsin; Schobert and Lanyi, 1986); (4) competitive interactions of anions having different effectiveness (acetoacetate decarboxylase: Fridovich, 1963; α-amylase:

Levitzki and Steer, 1974; angiotensin converting enzyme: Bünning and Riordan, 1983); (5) the possible interaction of the anion with a metal center (carbonic anhydrase: Lindskog, 1983); (6) the involvement of a heterolytic splitting of water (many anion-requiring enzymes are hydrolases) and protonation steps (phenol hydroxylase: Detmer and Massey, 1984); (7) a dual requirement for an anion and Ca^{2+} and a structural effect of Cl^- (α-amylase: Levitzki and Steer, 1974).

Aside from these characteristics shared with other systems, the relation of the photosynthetic water-oxidizing system to Cl^- is also unique in some ways: (1) there are perhaps two types of Cl^- interactions, at least one of which apparently having gross structural implications; (2) Cl^- binding is controlled by groups with a rather low pk_a; (3) Cl^- actions are modulated by the presence of ancillary polypeptides; (4) Cl^- prevents in seemingly competitive fashion the approach to some critical site not only of other anions, but also of uncharged Lewis bases like amines; (5) Cl^- action is impeded by SO_4^{2-} with a pH dependence that suggests that its interaction is made possible by a deprotonation event rather than, as is usually the case (Koenig *et al.*, 1980; Knauf and Grinstein, 1982), the provision of an additional protonated anion-binding group; (6) Cl^- binding varies during the succession of intermediate reaction steps preceding product formation.

The features listed above provide a checklist for any attempt to add another model describing photosynthetic water oxidation and Cl^- action to the many already existing in the literature. Some general implications will be discussed here.

It is not improbable that Cl^- participates directly in the mechanism of charge acquisition and use by the water oxidase. Accepting the notion that the critical steps of water oxidation involve Mn, such function of Cl^- in the catalytic mechanism implies some type of interaction with the metal center. A tight ligation of Cl^- to Mn, however, is not well supported by the experimental evidence. While Cl^- binding appears to respond to the extra positive charge of the S_2 and S_3 conformations (Preston and Pace, 1985), kinetic data suggest a persistence rather than a neutralization of this charge in the microspace around the S complex (Brettel *et al.*, 1984). Hence, under conditions which keep Cl^- sequestered at the site of water oxidation, it may only become delocalized in the S_2 and S_3 states. It is important to realize, in this context, that the light dependence of Cl^- as well as Ca^{2+} binding might ultimately be explained on the basis of structural ramifications of the S-state transitions about which essentially nothing is known at this time (see Brudvig's contribution to this series). One type of structural modification may be associated with proton transfer in the course of water oxidation. This view is supported by our observation that a lower pH can fully substitute for light as a means to dissociate Ca^{2+} from its binding site at the water oxidase

(Homann, unpublished). Another structure-modifying event could be associated with the distribution of water molecules between the anion-associated protein surfaces and the Mn center.

It has been proposed that, by controlling the protonation at its binding site, Cl^- may regulate the protonation of the water ligands at the Mn center during their oxidation, and the approach of other ions (Govindjee *et al.*, 1985; Homann, 1985). Dismukes (1986) has discussed relevant ligand exchange effects on the redox potential of Mn which are in line with the properties of the "Σ" states seen in the thermoluminescence experiments. His observation that S_1 (Σ_1?) of Cl^--depleted preparations reverts to an S_0-like condition may be related to the modification of a protonation equilibrium ("alkalinization" effect). Indeed, a similar deactivation of S_1 has been seen when the medium pH was raised (Plijter *et al.*, 1986).

A second aspect of Cl^- action is structural. Expressions of this role are the protection afforded by Cl^- against inactivation of PSII by heat, and its ability to promote recovery therefrom (see review by Critchley, 1985); the NaCl requirement of the paramagnetic coupling of one Mn pair in preparations lacking all three extrinsic polypeptides (Mavankal *et al.*, 1986); and perhaps the observation that, in PSII membranes depleted of Cl^- by NaF, the reappearance of the EPR multiline signal required the addition of less Cl^- than the restoration of water-oxidizing activity (Damoder *et al.*, 1986).

A fascinating feature is the apparent interplay between the various constituents of the water-oxidizing complex in molding the active site. On the one hand, the assembly of the 23- and 17-kDa polypeptides into the water-oxidizing complex requires the prior insertion and functionalization of Mn. On the other hand, these polypeptides are often labilized during extraction of Cl^-, but rebind when Cl^- is added. Hence, Cl^- could be the link between Mn and the binding of these polypeptides. The conceptual dilemma is obvious: either we deal with a large-scale response to a localized structure parameter, or Cl^- modulates protonation equilibria and hydration of polypeptide surfaces thereby regulating polypeptide conformations and interactions. These, in turn, may be important for proton conduction from the active site to the thylakoid lumen (Homann *et al.*, 1983). It is important to remember, in this context, that *in situ* the full complement of constitutents of the water-oxidizing site is located in a carefully controlled aqueous space of very limited dimensions, yet studded with functional groups of proteins and lipids.

The interactions of Mn, Ca^{2+}, and the 33-kDa polypeptide are similarly puzzling. The available data on oxidant storage after removal of Ca^{2+}, or the 33-kDa polypeptide, are consistent only inasmuch as they suggest an impairment of electron transfer somewhere between S_3 and Z^+. An oxidation of the Mn center(s), however, could not be verified by normally applicable direct methods. Presumably, if oxidizing equivalents are stored on Mn, the

charge distribution among the functional bound Mn ions or Mn pairs, and their ligands, are modified due to structural implications of the removal of the polypeptide and/or Ca^{2+}. For the former, an effect on Mn binding is well established, but our knowledge about relevant actions of Ca^{2+} is limited to its requirement during the photoactivation of Mn. In this case, the role of Ca^{2+} could be to assure proper removal of electrons from the nascent Mn center(s). An organizing function of Ca^{2+} for the Mn binding site as in concanavalin-A (Ghanotakis *et al.*, 1985b) might well be involved, either directly or indirectly.

While Ca^{2+} action at the water-oxidizing site is, by and large, very specific for this ion, the function of Cl^- can be taken over by several other monovalent anions. Their lesser degree of effectiveness is reflected by a lowered quantum efficiency (Homann, 1985), lower maximal rates of oxygen evolution, and a slowed $S_3 \rightarrow (S_4) \rightarrow S_0$ transition (Sinclair, 1984). Such responses to substitutes support the notion that in intact water-oxidizing systems the main function of the anions is in the catalytic mechanism which, when inefficient, leads to diversions and losses of oxidizing equivalents. Significantly, the lower efficiency of NO_3^- has been found to be coupled with an inability to save S_1 from inactivation (Damoder *et al.*, 1986) and a poor retention at the water-oxidizing site (Homann, 1985). This may be a consequence of size, geometry, charge density, and hydration (Critchley *et al.*, 1982; Damoder *et al.*, 1986). After removal of the 23-kDa polypeptide, Cl^- action is mimicked by other anions with even lower effectiveness (Homann and Inoue, 1985). The reason could be the limitation of water-oxidizing activity by the surface-directed rather than the site-directed anion actions which would be affected in more profound ways by the chaotropic properties of the anion.

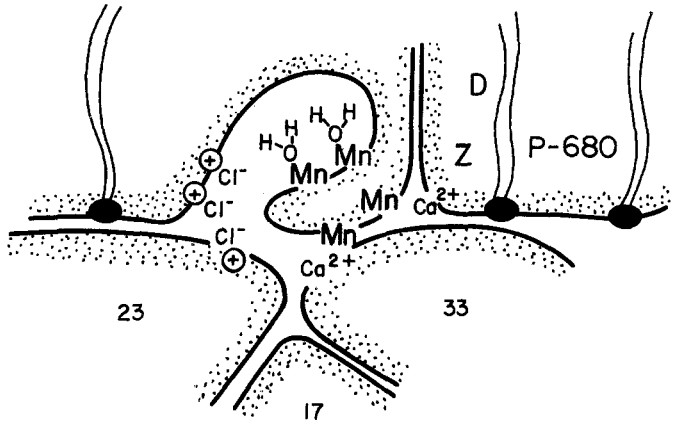

Fig. 2. Schematic presentation of a possible organization of the water-oxidizing site of photosystem II on chloroplast thylakoids (see text). New data (Homann, unpublished) suggest that the cationic Cl^- binding sites are flanked by protonatable anionic groups of $pK_a < 5$.

A hypothetical view of the organization of the water-oxidizing site is presented in Fig. 2. The underlying concepts were developed from the information contained in this article, and the experimental approaches to a topography of that region by Ljungberg *et al.*, (1984) and Isogai *et al.*, (1985).

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