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FUNCTION OF CHLORIDE IN WATER OXIDATION IN PHOTOSYNTHESIS

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ABSTRACT

The function of chloride (Cl^-) in the process of photosynthetic water oxidation is still poorly understood. We present here a mini-review of our current understanding of this phenomenon. Chloride is a well-established cofactor of oxygen evolution. Its site of action is on the electron donor side of photosystem II (PS II) between the water oxidation complex (WOC) and the electron donation sites of external donors such as hydroxylamine (NH_2OH), diphenylcarbazide (DPC) and catechol. The effectiveness of anions supporting water oxidation is: $Cl^- \sim Br^- > NO_3^- >> I^- \sim ClO_4^- \sim CNS^- > HCO_2^- \sim ClO_3^- > F^- \sim BrO_3^- \sim CH_3CO_2^-$. Bicarbonate ($HCO_3^-$) that appears to be required on the electron acceptor side of PS II, is not very effective in replacing Cl^-. Sulphate (SO_4^{2-}) and HPO_4^{2-} are totally ineffective; F^- and OH^- may even be inhibitory. The relative effectiveness of the anions as activators of O_2 evolution is changed by the removal of extrinsic 17 and 23 kDa polypeptides. Anions in a similar, but not identical, order of effectiveness prevent dissociation of 17 and 23 kDa extrinsic polypeptides and protect thylakoids against mild heating. The size, ionic field and hydration energies of the anions seem to play significant roles. It appears that several (4 to 40) Cl^- ions are weakly and reversibly bound per WOC. Chloride removal from the PS II has a profound effect on its electron donor side: there is no O_2 evolution and the cycle of the so-called S-states of the WOC is arrested in an abnormal S_2 state in which two oxidizing equivalents are stored. Its aberrant nature is evident from its lowered oxidation potential and its inability to produce the typical multiline EPR signal from the functional Mn center. Replacement of chloride with bromide does not change the hyperfine structure of the multiline Mn signal. In the absence of functional anions, the susceptibility of the WOC to stress such as heat, NH_2OH and OH^- is increased and the association of the extrinsic 17 and 23 kilodalton (kDa) polypeptides is destabilized. After detachment of these polypeptides, [Cl^-] is no longer sequestered in the PS II complex and approximately ten times higher [Cl^-] must be in the medium for optimal activities; another ten-fold

increase of the Cl^- requirement is observed after removal of the third, 33 kDa extrinsic polypeptide. These phenomena are rationalized best by the existence of a multitude of anion binding sites. $^{35}Cl^-$ NMR has allowed a direct look at the interaction of Cl^- with WOC, and in fact, suggests that binding sites of various affinities exist. Removal of functional Mn seems to have little effect on these populations of binding sites; however, these sites are characteristically affected by the extrinsic polypeptides. In all likelihood, there are two binding domains: (1) intrinsic: on the PS II reaction center proteins D_1 and D_2 (perhaps on the lumenal histidines); and (2) extrinsic: on the extrinsic 33 kDa polypeptide (perhaps on lysines and/or arginines). Our hypothesis for the Cl^- action is: it facilitates the proton abstraction from substrate water during its oxidation to molecular O_2 ($2H_2O = 4H^+ + 4e^- + O_2$). This may be achieved by Cl^- causing an extensive rearrangement of H^+ accepting groups around the active site of water oxidase. As part of such a picture, we consider the possibility that Cl^- binds to a positively charged amino acid (N^+) which is close to an anionic or neutral amino acid (B^-) in the neighborhood of the substrate H_2O molecules. Binding to N^+ may change the pk_a of B^- such that its affinity to H_2O protons increases. In this hypothesis, release of H^+s is accompanied by the release of Cl^- ions.

INTRODUCTION

Chloride (Cl^-) is required for the growth of plants (Epstein 1972; Clarkson and Hanson 1980), but in nature or the field chlorine deficiency is never encountered. However, it can be easily induced in the laboratory. Deficient plants wilt and a build-up of free amino acids is observed in them (see e.g. Johnson et al. 1957). Chloride, by virtue of being a counterion to K^+, is known to contribute to the turgor of guard cells (Raschke and Schnabl 1978; Schnabl 1978). In Beta vulgaris, chlorine-deficiency causes reduced leaf growth and partial chlorosis in the area of the veins (Terry 1977). Usually 50 - 500/umoles of chlorine/g dry weight is present in plants; deficiency symptoms can be observed when it reaches a value of 2 - 20 /umoles/g dry weight. However, this deficiency is not enough to affect photosynthesis since Cl^- is tightly sequestered at its binding site in chloroplasts. Larkum (1968) found Cl^- to be concentrated in chloroplasts of the alga Iolypella intricata.

In addition to the various effects on the growth of plants, Cl^- is known to be required for the functioning of the oxygen-evolving complex, i.e. water oxidase of photosystem II (PSII). This requirement is not specific to Cl^- as some other anions can replace it with a

varying degree of efficiency, e.g.: $Cl^- > Br^- > NO_3^- > I^- > ClO_4^- \sim HCO_3^-$. The influx and efflux of Cl^- at the oxygen-evolving complex may be governed, in a complex manner, by the concentrations of this and other anions and of cations (including protons) in various compartments. (For a discussion of influx of Cl^- in intact systems, see Cram 1973; Smith 1973; and Sanders and Hansen 1980.) Since some anions can replace Cl^-, the oxygen-evolving complex might continue to function in vivo even in the absence of Cl^-, provided other substituents are available. In this minireview, we shall discuss the mechanism of action of Cl^- in PS II. For earlier discussions, see Izawa et al. (1983), Homann et al. (1983), Govidjee et al. (1983, 1985), Critchley (1985), Homann (1987a), Coleman and Govindjee (1987a,b), Gowindjee (1988), and Coleman (1988).

HISTORICAL

Warburg and Lüttgens (1944, 1946) (Warburg 1949) discovered that various anions (Cl^-, Br^-, I^-, and NO_3^-) stimulated oxygen evolution by the Hill reaction in water-washed broken chloroplasts; Cl^- was the most effective anion, and SCN^-, SO_4^{2-} and HPO_4^{2-} were ineffective. The involvement of Cl^- in the O_2 evolution process during the Hill reaction was first clearly shown by Gorham and Clendenning (1952). Research on the role of anions in photosynthesis did not receive much recognition for a long time. For example, Gaffron (1960) in his famous review of the field of photosynthesis did not even mention it. Bové et al. (1963) showed that chloride was needed for noncyclic, but not cyclic, electron flow in chloroplasts. Rabinowitch and Govindjee (1969) in their little book wrote one sentence about it. In 1969, Hind et al., Heath and Hind, and Izawa et al. brought the problem back into focus. Yet, Avron (1975) devoted only two sentences of his review to Cl^-. The excellent work of Kelley and Izawa (1978), finally, provided a detailed scientific basis for the involvement of Cl^- (or its substitutes) on the electron donor side of photosystem II. However, the molecular mechanism by which Cl^- activated O_2 evolution remained a mystery.

SITES OF ACTION AND BINDING

Figure 1 (Govindjee 1988) suggest that the site of action of Cl^- is on the electron donor side of photosystem II somewhere between oxygen evolution and the electron donor Z, that has been suggested to be tyrosine 160/161 in the D-1 polypeptide (Debus et al. 1988; Vermaas et al. 1988) on the reaction center II (Nanba and Satoh 1987). Evidence for this site of action came from fluorescence studies which showed that the variable chlorophyll a fluorescence was absent in chloride-depleted samples and could be restored not only by the addition of chloride or bromide, but also by electron donors to Z such as hydroxylamine, catechol or diphenylcarbazide (Heath and Hind 1969a; Critchley et al. 1982). Kelley and Izawa (1978) established, through a study of the partial electron transport reactions, that electron flow from added electron donors to Z was possible in chloride-depleted samples.

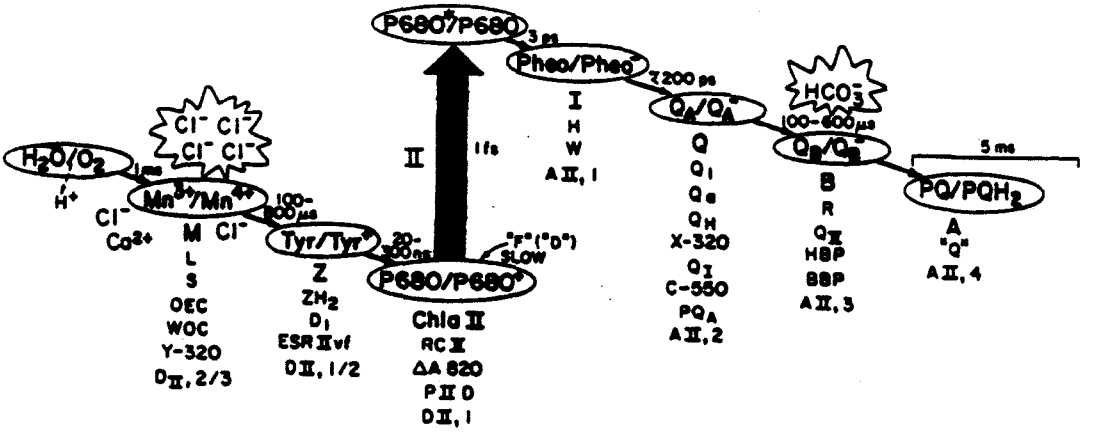

Fig. 1A. Electron flow in photosystem II. The scheme shows the main intermediates; alternate names used by various authors are also listed below the intermediates. Estimated or measured times of reactions are also listed. OEC = oxygen evolving complex; WOC = water oxidizing complex; HBP = herbicide binding protein; BBP = bicarbonate binding protein. AII,1,2,3 and DII,1,2,3, etc. refer to electron acceptors and donors of photosystem II. "F"("D") is a slow donor, shown to be a tyrosine. The identity of Z to be a tyrosine is only a speculation. The Fe of $Q_A \cdot Fe \cdot Q_B$ complex has been shown to be equivalent to Q-400 that may also be equivalent to Q_L, Q_2 or X_a (Modified after Govindjee 1984).

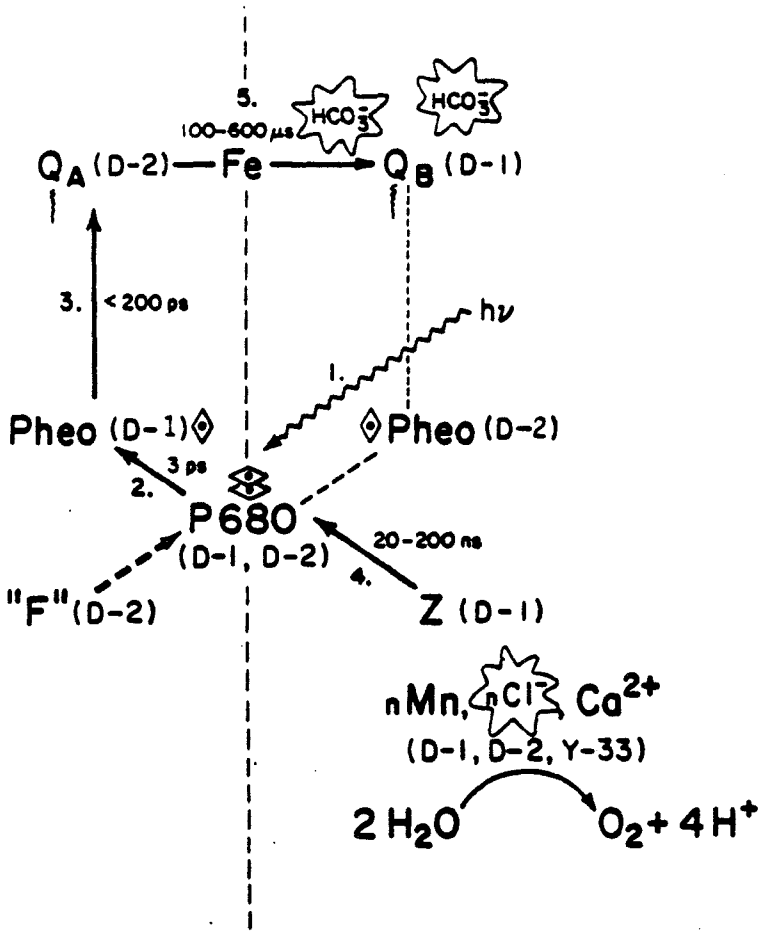

Fig. 18. Schematic organization of the reaction center of photosystem II. Intermediates are shown in Bold; their location on the individual polypeptides are given in parenthesis. Y-33 is an extrinsic polypeptide of 33 kDa mass; D-1 and D-2 are intrinsic polypeptides, also of 32-33 kDa masses. (For composition of Reaction Center, see Nanba and Satoh 1987). 1, 2, 3, 4 and 5 represent the order in which reactions may occur following a flash.

Sinclair (1984), on the basis of kinetic data, suggested that there may be, at least two separate binding environments for Cl^-. Figure 2 shows a schematic diagram of the two proposed binding niches of chloride in photosystem II (Coleman and Govindjee 1987b): (a) the extrinsic on the extrinsic 33 kDa polypeptide (often called Y-33; see Andersson and Åkerlund 1987) on the lysines and the arginines; (b) the intrinsic on the lumenal portions of the D-1 and D-2 polypeptides -- most likely on the histidines (e.g., H-337 on D-2, and H-332 and H-337 on D-1). We expect the intrinsic chloride sites to be close to

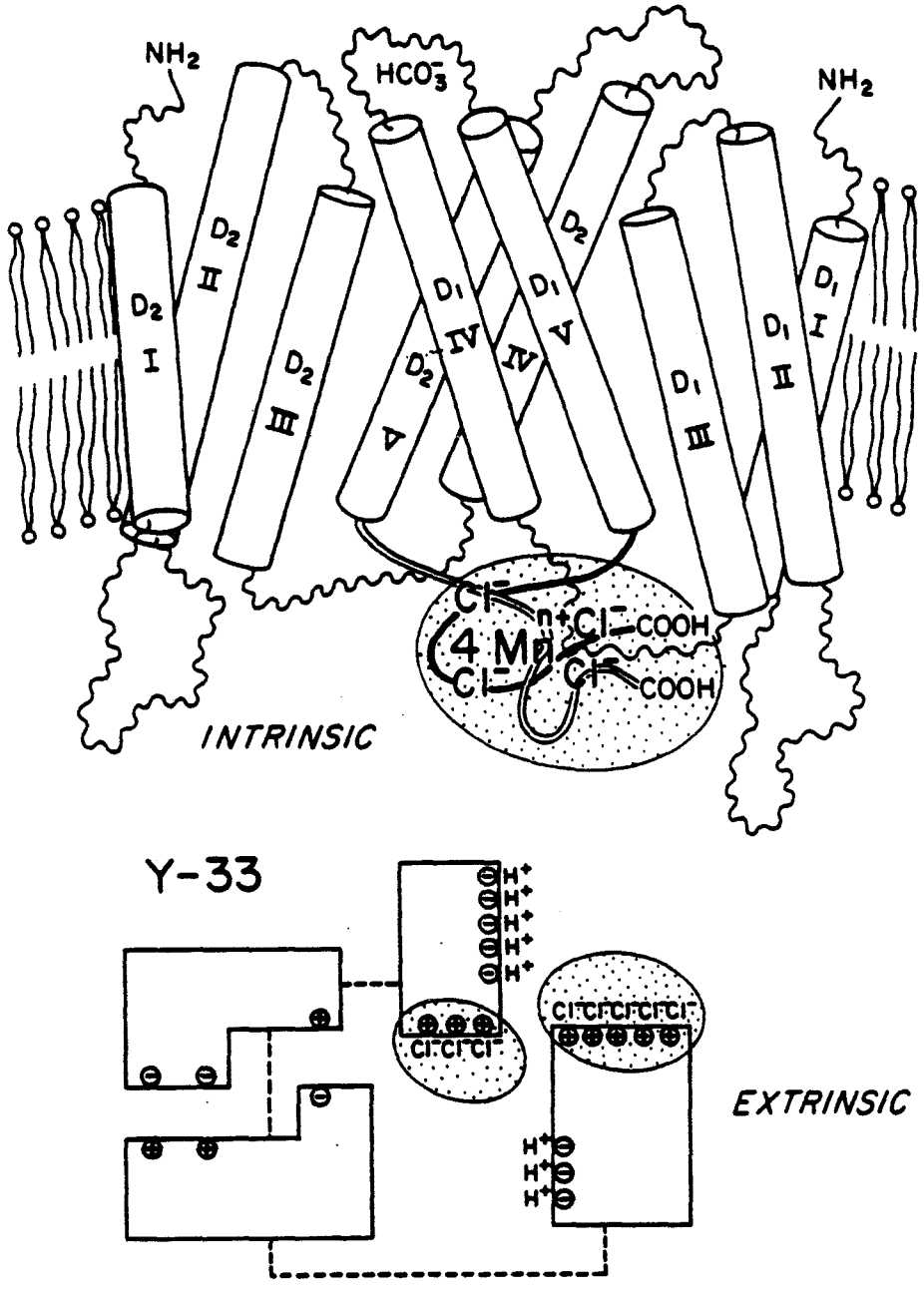

Fig. 2. Schematic diagram of possible binding sites for chloride. (Top): Intrinsic on the lumenal loops of D-1 and D-2 polypeptides (see text) and (bottom): extrinsic: on the Y-33, extrinsic polypeptide with a molecular mass of 33 kDa (see text; based on ideas presented by Coleman and Govindjee 1987b).

the Mn sites since both are involved in O_2 evolution, and Cl^- removal affects the EPR spectrum of the Mn center in its S_2 state (Beck and Brudvig 1988). It is suggested that 4 Mn atoms/reaction center II

form two pairs in two different environments (Kambara and Govindjee 1985; Andersson and Åkerlund 1987; Renger 1987), but no definite information is available about the actual locations.

Coleman and Govindjee (1987b) have considered one possible model of how Mn can be accommodated between D-1 and D-2. It is known that the electron donor side of PS II is on the inner side of the thylakoid membrane (see e.g. Zilinskas 1975; Diner and Joliot 1977; Andersson and Åkerlund 1987). Significantly, the lumen exposed portions of D-1 and D-2 are different from those of the L and M subunits of bacterial reaction centers that do not evolve O_2 (Trebst 1986, 1987) and are conserved among various O_2 evolving organisms including cyanobacteria. Thus, Coleman and Govindjee (1987b) suggested 4 possible Mn binding sites provided by the negatively charged amino acids available on the 4 luminal segments. Distances between Mn atoms larger than 0.33 nm would be inconsistent with the existing X-ray absorption data (see reviews by Babcock 1987; Brudvig 1987; Renger 1987; Yachandra et al. 1987; R. Prince, personal communication 1988). Thus, if Z is indeed tyrosine-160 (Y on D-1, helix III), it would be convenient if Mn was nearby, e.g., on the lumenal portions connecting D_1-I and D_2-II and/or on the two loops with COOH ends (attached to D-1-V). Since the X-ray structure and distances within the reaction center of PS II are totally unknown, none of the possibilities can be ignored.

Whether Cl^- is directly ligated to Mn (Bové et al. 1963; Critchley and Sagerson 1984; Sandusky and Yocum 1983, 1984, 1986) or simply bound electrostatically in the neighborhood has not been satisfactorily resolved. Direct binding of chloride to manganese appears to be contradicted by the absence of an anion effect on the hyperfine structure of the Mn multiline EPR signal when chloride is replaced by bromide (Damoder et al. 1986; Yachandra et al. 1986a; Mavankal et al. 1986). Furthermore, the X-ray absorption measurements exclude chloride from the first coordination sphere of Mn in the S_1 and S_2 states of WOC (Yachandra et al. 1986b, 1987). Coleman et al. (1987c), furthermore, were unable to observe any significant changes in the linewidth of $^{35}Cl^-$NMR of photosystem II membranes upon removal of Mn by hydroxylamine treatment, and it is not difficult to remove

chloride while retaining Mn. However, inhibitory amines (NH_3, Tris) seem to compete with Cl^- for a binding site (Sandusky and Yocum 1983, 1984, 1986) but this competition, apparently is not on the functional Mn atoms (Beck and Brudvig 1988). Of course if Cl^- ions function to stabilize positive charges on Mn, Cl^- might be held in place only by electrostatic forces.

SPECIFICITY AND EFFECTIVENESS

Chlorine (Cl) belongs to the group VIII of the halogens. They are, in order of increasing atomic weight and size: fluorine (F_2; 18; covalent radius: 0.071 nm), chlorine (Cl_2; 35; 0.099 nm), bromine (Br_2; 79; 0.114 nm), iodine (I_2; 127; 0.133 nm) and the volatile astatine (At_2, 190). Generally, the stability of halide complexes decreases in the series $F^- > Cl^- > Br^- > I^-$, but with some metal ions it may be opposite. Halides form bridges; bridges with two halogen atoms are most common; with Cl^- and Br^-, bridges are often bent, and a metal may be coordinated with as many as 5 halide atoms. Fluoride is highly reactive due to its small size and high electronegativity. For basic information about halide chemistry, see e.g., Cotton and Wilkinson (1980) and Downs and Adams (1975).

With respect to their action on macromolecules in aqueous systems, the halides and other ions are arranged in what is called the lyotropic or Hofmeister series in reference to its discoverer, the physiological chemist Franz Hofmeister (see Collins and Washabaugh 1985). The order in the series does not simply depend on the individual ion properties but on forces developing between water, the ions, and the macromolecules. Although the order is not fixed, it is usually F^-, $SO_4^{2-} > Cl^- > Br^- > NO_3^- > ClO_4^- \sim SCN^-$, from structure stabilizing "cosmotropes" (F^-, SO_4^{2-}) to denaturing "chaotropes" (CLO_4^-, SCN^-) (Collins and Washabaugh 1985).

In photosystem II, the effectiveness of anions in stimulating electron flow follows the series: $Cl^- > Br^- > NO_3 > I^- > ClO_4^-$; SO_4^{2-} and HPO_4^{2-} are ineffective; while F^- and OH^- are usually inhibitory. Some investigators, however, have found F^- more effective than ClO_4^- as a substitute for Cl^- (see Kelley and Izawa 1978; Critchley et al. 1982). Even so, the activating anion action in PSII clearly is <u>not</u>

of a lyotropic nature.

Krishnan and Mohanty (1984) showed that Cl^- can protect thylakoids against mild heating and Tris-washing. Coleman et al. (1984) in their study of anion specificity for protection against damage by mild heating found that Cl^- was only slightly better than Br^-, and NO_3^- was as ineffective as SO_4^{2-}. Thus, the series was: $Cl^- > Br^- >> SO_4^{2-} \sim NO_3^-$. Critchley et al. (1982), on the other hand, found a dramatic difference between Cl^- and Br^- in some halophyte (A. marina) thylakoids (later shown to have lost 17 and 24 kDa polypeptides, unpublished observations).

Homann and Inoue (1986) have clearly shown that the anion selectivity in the activation of O_2 evolution depends on the polypeptide status of the sample. In normal samples, the series is Cl^- (100) $\sim$ Br^- (95) > NO_3^- (65) >> I^- (40) > ClO_4^- = CNS^- (30) > HCO_2^- (15) $\sim$ ClO_3^- (15) > $F^- \sim BrO_3^- \sim CH_3CO_2^-$ (< 5), the numbers in brackets represent effectiveness in supporting O_2 evolution. These are changed by the removal of 17 and 23 kDa extrinsic polypeptides to Cl^- (100) >> Br^- (55 $\sim$ NO_3^- (50) >> ClO_4^- (25) > I^- (15) $\sim$ CNS^- (15) $\sim$ HCO_2^- (15) > $CH_3CO_2^-$ (10) > F^- (5). The changing anion effectiveness suggests that the actions of different anions are modulated by changes in the selectivity of the binding sites, which in turn, depend on the presence or the absence of various polypeptides; heating may cause additional conformational perturbations (Nash et al. 1985; Coleman et al. 1988). (For a review of other systems, see Wright and Diamond 1977.)

Homann (1988b) has reported that mM SO_4^{2-} (and divalent cations) enhance the dissociation of 17 and 23 kDa polypeptides in Cl^--free media at pH 7, and that this dissociation is proposed by relatively low concentrations of certain monovalent anions in the order Cl^- = $Br^- > NO_3^- > F^- > ClO_4^-$. This series was quite similar to the series that stimulates O_2 evolution and suggests that a specific binding of Cl^- (or substitutes) organizes the protein surfaces and/or adjacent water layers in the water oxidizing complex in a way that not only is essential for the catalytic mechanism, but also stabilizes its assembly. In contrast, at higher (molar) concentrations, F^- and SO_4^{2-} stabilized the binding of the 23 kDa polypeptide, and Cl^- and Br^- were dissociating agents in accordance with lyotropic series (see Fig.3).

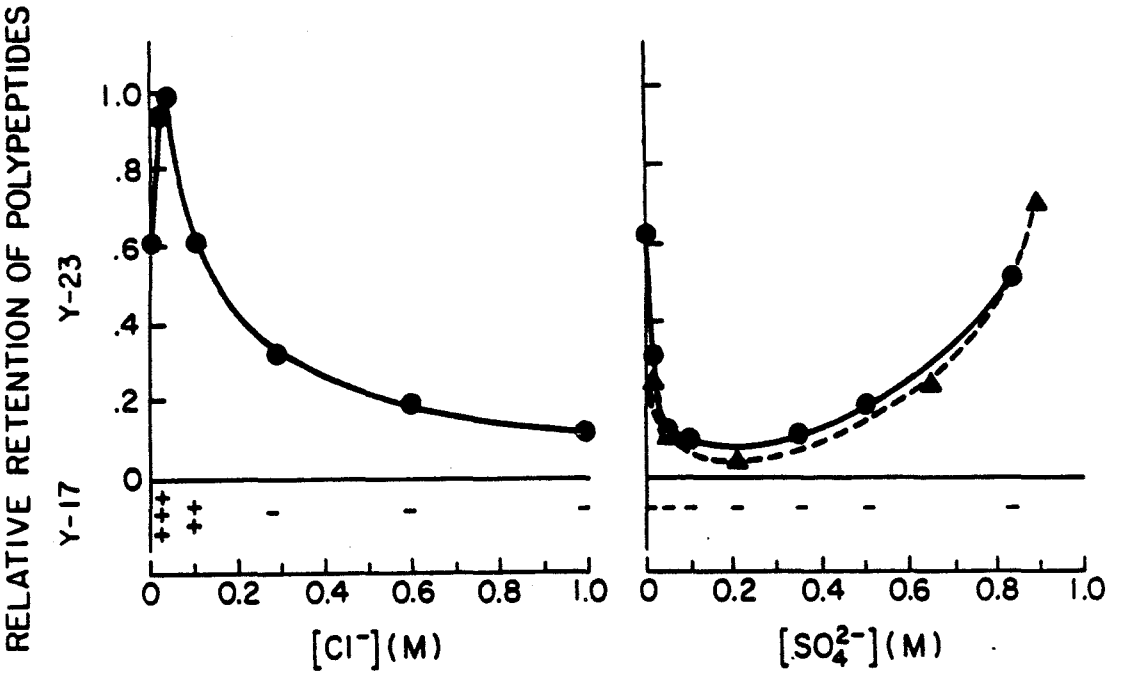

Fig. 3. Dependence of the retention of the 17 and 23 kDa polypeptides on the concentration of NaCl (left) or Na_2SO_4 (right) in the incubation medium (1 h, 5 °C). 0.4M sucrose; 40 mM Ma-Mops, pH 7.0 or pH 6.85 (▲--▲); 400/um Chl/ml (after Homann 1988).

Hind et al. (1969) have pointed out that the ionic volume may be the critical factor in dictating the effectiveness of an anion; that is, smaller the anion, the greater the activity. Perhaps, a "gate" or "pocket" exists for the entry of the anion. Figure 4 shows that Cl^- volume of 0.025 nm^3 is optimal for activity in spinach thylakoid. Critchley et al. (1982) suggested that ionic volume is not the only factor because F^-, OH^- and acetate (Ac^-) that have smaller volumes than Cl^- gave low or no stimulation of electron flow (for function of HCO_3^- on the electron acceptor side, see Govinjee and Eaton-Rye 1986). A plot of the effectiveness of the anion as a function of ionic field (= $\dot{Q}_A/e_s r_A 2$, where $\dot{Q}_A$ is the anion charge, r_A is Pauling radius and e_s is the differential dielectric constant of water) showed that the effectiveness of anions decreases with decreasing field strength. However, anions with large anionic field (SO_4^{2-}, Ac^-, PO_4^{3-}, OH^- and F^-) were also relatively ineffective in stimulating electron flow. Thus, any proposed mechanism of the Cl^- (or substitute) function must include consideration of the anion volume,

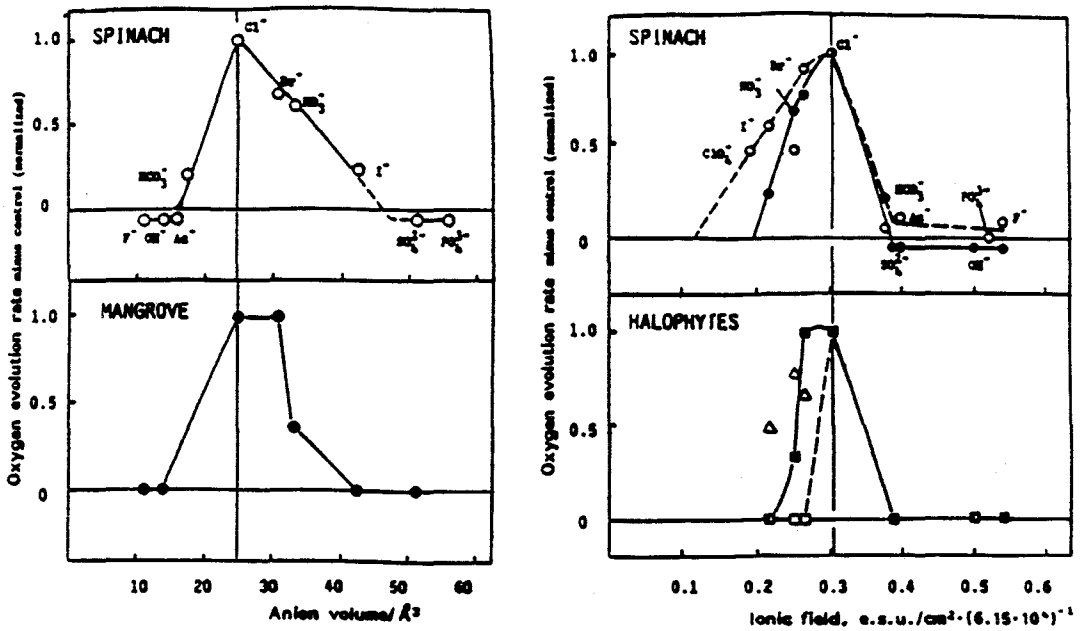

Fig. 4. Left: Hill reaction rates versus the anion volume. Spinach data are for pH 7.2, and Mangrove data are for pH 7.8. Right: Hill reaction rates versus the ionic field. Dashed curve for spinach is for pH 8.3, and solid for pH 7.2. Dashed curve (and open squares) for halophytes refers to that for Avicennia marina, and the solid curve is for Atriplex tripolium (triangles) and Avicennia germinans squares), all at approximately pH 7.8 (after Critchley et al. 1982).

ionic field and hydration energy of the anion, and the ability to organize the structure (protein surface, etc.). Further research is needed to unravel the interrelation between the mechanisms underlying the anion stimulation of O_2 evolution, and the protection by anions against heat damage and release of extrinsic polypeptides.

BINDING AND BINDING SITES

It appears that Cl^- (or substitutes) are necessary for the binding of 23 kDa polypeptide (Homann 1987b, 1988b,c) and that 23 kDa polypeptide helps keep the Cl^- sequestered or bound. Miyao and Murata (1985) have called this polypeptide "Chloride concentrator". Figure 5 shows the interaction of Cl^- and 23 kDa polypeptide restoring O_2 evolution. Considering the mutual stabilizing effect between Cl^- and 23 kDa polypeptide, it appears that usually both cooperate in organizing the water oxidase and in providing the appropriate structure for the enzymatic activity. However, we consider it quite feasible

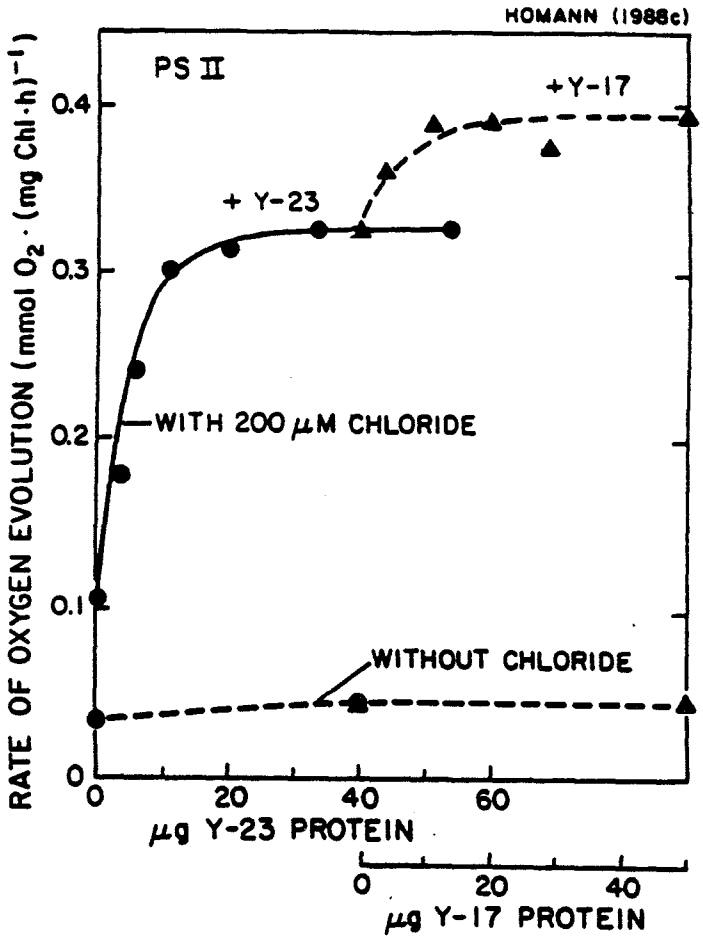

Fig. 5. Dependence on the oxygen evolution activity of photosystem II membranes depleted of their Y-17 and Y-23 on the amount of resupplied polypeptides in the presence and the absence of 200 μM NaCl (after Homann 1988c).

that Cl^- may serve, in addition, a more intricate catalytic role in water oxidation. Whether the structural and the catalytic effects are expressions of the same interactions or whether they are separate from one another, remains to be discovered.

It has been established that removal of 23 kDa polypeptide increases the Cl^- requirement by ten times and that a further removal of 33 kDa polypeptide increases it by another ten fold (Andersson et al. 1984; Miyao and Murata 1985; Ghanotakis et al. 1985; and Homann 1988c; for other references, see citations in Coleman et al. 1987c). The relationship of 17 kDa polypeptide to the role of Cl^- in PSII remains unclear (Akarobi et al. 1984; Andersson et al. 1984; Miyao

and Murata 1985; Homann 1988c) but appears to be minor (Fig. 5). It is our general impression that the binding sites of Cl^- are not on 17 and 23 kDa polypeptides although the latter, at least, may be indirectly involved.

Number of binding sites

If Cl^- were just a ligand of catalytic Mn, we would have expected numbers of Cl^- bound per reaction center to be 4, 8, 20, etc. since there are 4 Mn atoms per reaction center. It is, however, difficult to measure the number of only the so-called catalytic "Cl". Using radioactive ^{36}Cl, Theg and Homann (1982) obtained a value of $\sim$7 Cl^-/600 Chl in spinach thylakoids. Izawa, S. (personal communication, 1984) obtained a value that ranged from 4 to 40 Cl^-/600 Chl. Using $_{35}Cl$ NMR line-broadening measurements and several assumptions, Govingjee et al. (1983) and Baianu et al. (1984) estimated a value of 20-40 Cl^-/600 Chl in thylakoids from halophytes, which may have been lacking in the two extrinsic polypeptides, also suggesting that multiple binding sites exist for Cl^-.

NMR studies and binding sites

The first positive observation, using NMR technique, was with ^{19}F relaxation rate measurements on Cl^--depleted samples to which NaF was added (Govindjee et al. 1978). Although F^- does not support O_2 evolution, treatments that removed or chemically reduced manganese decreased or increased relaxation rates suggesting that F^- was bound at a site that was under the influence of Mn.

The [Cl^-]dependence of excess ^{35}Cl NMR linewidth data (i.e. corrected for that in solution) in halophytes (Baianu et al. 1984) and in spinach[+] (Coleman et al. 1987a,b,c, 1988) show a general pattern:

[+] ^{35}Cl NMR data on spinach thylakoids and Photosystem II membranes were obtained on an NMR instrument at the University of Illinois at Urbana (NSF-250; 250 MHz homebuilt NMR spectrometer; spectra obtained at 24,508 kHz using a 33 μs 90^0 pulse and 360 ms recycle time; signals detected in quadrature with 32k data points and a spectral width of I25,000 Hz; signals were then block averaged (500 scans per block) and transferred to a Nicolet 1180 E Computer).

there is a large excess linewidth at the low [Cl^-] That decreases with increasing [Cl^-]. Such a curve suggests that the tightly binding sites are filled at low [Cl^-]. These tightly binding sites at the lowest [Cl^-] are likely to be related to the catalytic function. Chloride affinity at these sites seems to decrease when all the extrinsic polypeptides including the 33 kDa polypeptide are removed: instead of the largest linewidth being at 0.1mM Cl^-, it is at 0.5mM (Coleman et al. 1987c). This is consistent with the idea that the [Cl^-] requirement for the O_2 evolution activity of such preparations is extremely high. Interestingly, a removal of Mn by NH_2OH, that eliminates O_2 evolution, only slightly alters the [Cl^-] dependence of the ^{35}Cl-NMR linewidth (Coleman et al. 1987c). Future investigations will have to analyze any direct correlation that exists between O_2 evolution and $^{35}Cl^-$ detected Cl^- binding at the high affinity sites.

Multiple sites

In addition to the high affinity site mentioned above, it appears that ^{35}Cl NMR data show the existence of other sites. Plots of ^{35}Cl-NMR linewidth, corrected for that in solution, show several maxima (see Fig. 6) that occur at roughly the same [Cl^-] as intermediary plateaus in a plot of Hill activity (at low light intensities) versus [Cl^-], suggesting a possible correlation between the two measurements (Coleman et al. 1987b). Nevertheless, Coleman and coworkers were concerned about a possible artifactual production of such maxima (Govindjee 1988). However, they could not observe them in measurements of $^{35}Cl^-$NMR linewidths with (a) Cl^-/buffer solutions containing0.1, 1.0 and 10mM Cl^-; these revealed linewidths of exactly the same values (differences of 1Hz or less); and (b) highly purified bovine serum albumin (a Cl^- binding protein with multiple sites) in the same [Cl^-] range as used for PSII membranes (Coleman et al. 1987b).

The observed correlation of the measured linewidth maxima with the Hill activity of the preparations (Coleman et al. 1987b) indicated that whatever structures contribute to the linewidth also contribute to the overall Hill activity. Furthermore, each maximum appeared to have a different degree of sensitivity to inactivations of the Hill

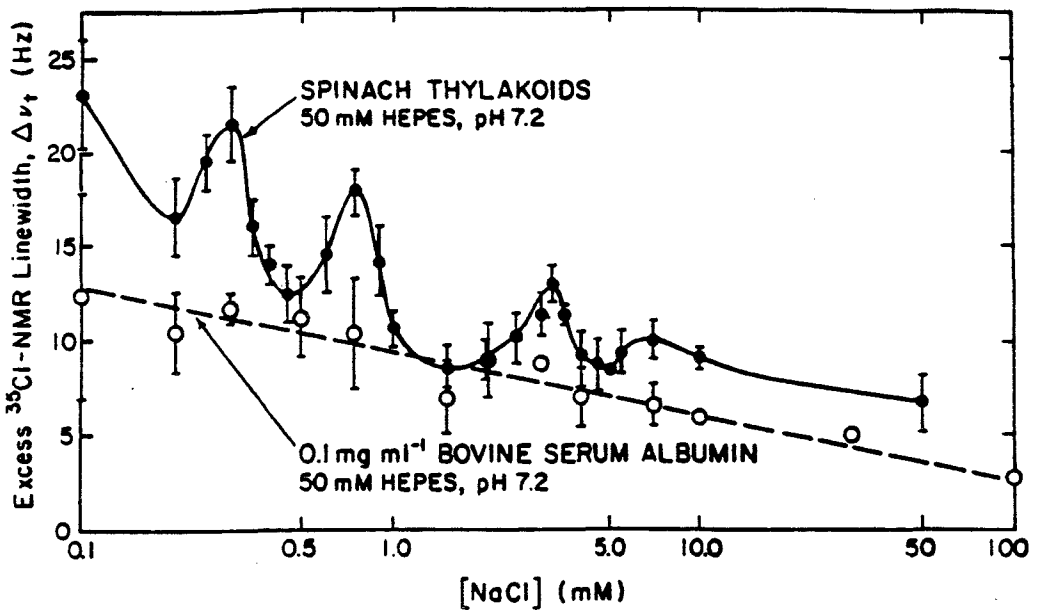

Fig. 6. ^{35}Cl-NMR binding curve for thylakoid membranes and for bovine serum albumin (BSA). The buffer was 50 mM HEPES at pH 7.2; the chlorophyll concentration was 0.5 mg. ml^{-1}. BSA was dissolved at a concentration of 0.1 mg. ml^{-1} in 50 mM HEPES at pH 7.2 $\Delta\nu_t$ (Hz) = excess line width; it is equal to $\Delta\nu_{obs}$ (observed linewidth at half-maximum intensity) minus $\Delta\nu_f$ (linewidth at half-maximum intensity for Cl^- solution. $\Delta\nu_{obs} = \Delta\nu_{bound} f_{bound} + \Delta\nu_f (1-f_{bound})$, where $\Delta\nu_{bound}$ is the weighed average of the contributions from Cl^- in the bound state, $\Delta\nu_f$ is the same in the free state, and f_{bound} is the fraction bound. Since $\Delta\nu_b$ (approximately 10kHz) is very much larger than $\Delta\nu_f$ (approximately 12-30 Hz, depending on the viscosity) and $f_{bound} << 1$ (for a dilute protein solution), $\Delta\nu_t = \Delta\nu_{bound} f_{bound}$ (after W.J. Coleman, unpublished; Coleman et al. 1987a,b,c)

reaction (Coleman et al. 1987a,b,c, 1988) indicating that any one maximum reflected a discrete structural entity. The treatments were mild heating (Coleman et al.); alkaline Tris (to remove 17 and 23 and 33 kDa polypeptides), salt washing (to remove 17 and 23 kDa, and 33 kDa polypeptides) and hydroxylamine (to remove Mn) (Coleman et al. 1987a,c). When only the 17 and 23 kDa polypeptides had been removed by NaCl washing 3 of the 4 lost maxima could be restored by adding 2mM $CaSO_4$; such a response to Ca^{2+} demonstrated another correlation with the Hill activity of the polypeptide depleted preparations which typically is stimulated by adding Ca^{2+}. $^{35}Cl^-$NMR line-broadening in the presence of PSII membranes in the [Cl^-] range between 0.1mM and 0.3mM was eliminated by a very low concentration of Br^- (0.1mM);

the observed reduction in the height of the first linewidth maximum is also consistent with the competition between the anions for a limited set of binding sites.

It is considered possible that the $^{35}Cl^-$NMR maxima at higher $[Cl^-]$ than needed for activating O_2 evolution may reflect a regulatory, rather than a catalytic function of Cl^-. Mild heating (3 min., 30 °C) eliminated the same high $[Cl^-]$ "kinks" of maxima and minima of both $[Cl^-]$ dependence of the $^{35}Cl^-$NMR linewidth and the Hill reaction (Coleman et al. 1988), which were dramatically altered also by the removal of the "regulatory" proteins (18 and 24 kDa) and partially restored by the addition of Ca^{2+} (Coleman et al. 1987a,c). The above leads us to suspect that these are also manifestations of Cl^- organizing the surface of the involved proteins.

RELATION TO THE S-STATES OF THE WATER OXIDASE

We have already mentioned that Cl^- functions on the electron donor side of PSII between (or at the) charge accumulator "M" and "Z" where several artificial electron donors are able to donate electrons. Hence, the question is whether Cl^- deficiency affects the "charge accumulating" process. The charge accumulator "M" (possibly Mn itself) exists in several redox states, labeled as S-states (see e.g., Joliot and Kok 1975). In darkness, "M" is in state "S_1", and the following sequence takes place in the first cycle in a dark-adapted sample:

$$S_1 \xrightarrow{^1h\nu} S_2 \xrightarrow{^2h\nu} S_3 \xrightarrow{^3h\nu} S_4 \xrightarrow{2H_2O,\ O_2} S_0$$

And the following sequence occurs in the subsequent cycle:

$$S_0 \xrightarrow{^4h\nu} S_1 \xrightarrow{^5h\nu} S_2 \xrightarrow{^6h\nu} S_3 \xrightarrow{^7h\nu} S_4 \xrightarrow{2H_2O,\ O_2} S_0$$

Here, $^n h\nu$ stands for a light flash. and S_n for a redox state "M", the higher n's representing higher oxidation states. Based on delayed light emission data (see Jursinic 1986), Muallem et al. (1980) and Muallem and Laine-Boszormenyi (1981) suggested that Cl^- depletion causes a defective oxidant storage characterized by high stability

of the "S" states in Cl^- depleted samples (Izawa et al. 1983). Govindjee et al. (1983) suggested that Cl^- was required at the steps that leave a positive charge on "M". Independently, Theg et al. (1984) and Itoh et al. (1984) measured the effect of Cl^- depletion on the S-state transitions and concluded that the system is blocked after two "holes" accumulate on the electron donor side; clearly, the $S_3 \longrightarrow S_0$ transition is unable to take place. Whether the two electrons are stored as S_2Z^+, or as a very unstable S_3 is not clear. On the basis of thermoluminiscence (Sane and Rutherford 1986) data, Homann et al. (1986) and Vass et al. (1987) suggested that Cl^- depletion generated an abnormal "S_2" state called "Σ_2" state. Several investigators, using a variety of approaches, have confirmed this conclusion. The abnormality of S_2 was confirmed by measurements on the multiline Mn EPR signals in Cl^- depleted samples (Damoder et al. 1986; Imaoka et al. 1986; Ono et al. 1986a, 1987a) and in those provided with substitute anions (Damoder et al. 1986; Ono et al. 1987b). This state does not produce the normal multiline ESR signal for Mn. However, the g=4.1 signal of the S_2 state is clearly present in these samples. Addition of Cl^- restores the normal S_2 signal. Rozsa and Demeter (1987), using thermoluminiscence measurements, suggested that Cl^- depletion blocks the conversion of S_2 to S_3, and it is proposed in agreement with oters (Theg et al. 1984; Itoh et al. 1984; Ono et al. 1986a) that the system remains "stuck" in Σ_2Z^+ state.

$^{35}Cl^-$ NMR binding studies as a function of flash number have suggested that Cl^- binds to $S_2 + S_3$ states only (Preston and Pace 1985). However, while consistent with predictions from the experiments just discussed, this binding may not reflect catalytically active Cl^- but anion binding due to changes in the conformation of the protein on which the charge accumulator resides.

MECHANISM OF CHLORIDE ACTION

In order to formulate a theory for the mechanism of Cl^- action, it is useful to keep in mind the following points (see also Coleman and Govindjee 1985; Homann 1987): (1) inactivation by Cl^- depletion is reversible; it appears that electrostatic interactions as well as

hydration and dehydration events may control binding (see e.g. Homann 1985); (2) Cl^- depletion is accelerated by incubating the thylakoids at high pH (Theg and Homann 1982; Izawa et al. 1983; Cole et al.1986); (3) Cl^- binding is pH dependent (C itchley et al. 1982; Homann 1985, 1988); (4) activation of the Hill reaction by added Cl^- shows hyperbolic kinetics, indicating saturation (Kelley and Izawa 1978; Baianu et al. 1984); (5) activation of the Hill reaction by anions is relatively, but not exclusively specific for Cl^- (see, e.g. Table I) and there is a competitive interaction of anions; (6) the pH optimum of the Hill reaction (Gorham and Clendenning 1952;Critchley 1983;Homann 1988a) as well as the polypeptide binding affinity (Homann 1987b) is shifted to more alkaline pH by Cl^- binding; this suggests that Cl^- addition is equivalent to an acidification.

To this check list, we must add (Homann 1987) the following unique properties of Cl^- of the water oxidazing steps: (1) There are two major binding domains for Cl^- or its substitutes (Coleman et al. 1987a); (2) Cl^- binding is controlled by a group with rather low pKa (5) (Homann 1988a); (3) there is modulation of Cl^- binding by the extrinsic polypeptides (Homann 1988b,c); both 17 and 23 kDa polypeptides are required for Cl^- sequestration, but neither of them need to bind Cl^- themselves; (4) Cl^- prevents binding of other anions and chemicals; (5) sulphate effects Cl^- binding at an elevated pH; its affects may merely be the facilitation of the dissociation of the 17 and 23 kDa polypeptides (Homann 1988b) and (6) Cl^- binding may vary during the S-state cycle.

Chloride binding does occur in dark-adapted samples (Baianu et al. 1984; Coleman et al. 1987b) and the exchange of Cl^- with the medium is rapid (Baianu et al. 1984; Itoh and Iwaki 1986). Baianu et al., on the basis of their data on $^{35}Cl^-$ NMR binding to halophytes, concluded that Cl^- in the membrane was in rapid exchange with the medium ($>>10^4 s^{-1}$) and that the binding was reversible and weak (12kJ/mol). These parameters need to be remeasured in samples with and without the extrinsic polypeptides. The binding seems to be modulated by the transitions of the WOC, i.e. the S-states. As mentioned earlier, there also is a clear interaction of Cl^- and polypeptide binding. Thus, the background level of Cl^- in medium and polypeptide samples

Table I. A representative Hofmeister Series, and rankings of anions according to their effectivenesses in actions on PSII (from Homann 1988b)

Response	Order of anion effectiveness[a]	Source
"Salting out" of a protein	$SO_4^{2-} \sim (F^-) > CH_3COO^- > Cl^- > Br^- > NO_3^- \gg ClO_4^- > I^- > CNS^-$	Collins and Washabaugh (1985), Von Hippel and Schleich (1969)
Activation of O_2 evolution in PSII membranes	$Cl^- \sim Br^- > NO_3 > I^- > ClO_4^- > CNS^- > (CH_3CO_2^-) = (F^-) = (SO_4^{2-})$	Homann (1985)
Same after removal of 17 and 23 kD polypeptides	$Cl^- > Br^- > NO_3^- > ClO_4^- > CNS^- \sim I^- \sim CH_3CO_2^- > (F^-) = (SO_4^{2-})$	
Stabilization of binding of 17,23 kD polypeptides	$Cl^- \sim Br^- > NO_3 > F^- > (ClO_4^-) > (SO_4^{2-})$	Homann (1988b)
Same, high ion concentrations	$SO_4^{2-} \sim F^- \gg (Cl^- > Br^- > NO_3^-)$	
Protection of PSII against Tris inactivation	$Cl^- > Br^- > NO_3 \gg HCO_2^-$	Sandusky and Yocum (1986)
Protection of PSII against heat	$Cl^- > Br^- > SO_4^{2-} \sim NO_3$	Coleman et al. (1984)
Labilization of a tightly bound Mn^- pool	$F^- > SO_4^{2-} > CH_3CO_2^- > Cl^- > Br^- > NO_3^-$	Hsu et al. (1987)

[a]Anions show in parenthesis had no, or opposite, effects.

may explain the conflicting results on the stimulation of O_2 by the various extrinsic polypeptides (Akabori et al. 1984; Andersson et al. 1984; Imaoka et al. 1984; Miyao and Murata 1985; Nakatani 1984; Homann 1988a).

Chloride not only affects the oxygen evolving mechanism, but also the reduction kinetics of $P680^+$ to P680 (Ono et al. 1986b); it is already known that this reduction step is pH dependent (Conjeaud and Mathis 1986; Schlodder and Meyer 1987). Furthermore, acetate that replaces Cl^- does inhibit the reduction of $P680^+$ to P680 (Saygin et al.

1986). These effects may be indirect and consequences of structural changes of the protein complexes on the electron donor side of PSII.

The relation between pH and Cl^- must hold the secret of the mechanism of Cl^- action. The pH profile of the anion requirement, and the correlation of the anion volume and the hydration energies of the activating anions with their effectiveness (Critchley 1985; Critchley et al. 1982) suggest that protonation of unique functional groups are critical for the mechanism of water oxidation. This supports the belief of Homann et al. (1983), Homann (1987) and Coleman and Govindjee (1985, 1987) that a major function of the anions is to organize the protein surfaces and suitable H^+ acceptor/donor groups at the active site of the water oxidase, thereby facilitating the proton abstraction from substrate water during its oxidation to molecular O_2.

Govindjee et al. (1983) have suggested that the catalytic function of the Cl^- was to act as a counterion to positive charges, arising from charge separation during light reaction and resident on the oxygen evolving complex (Renger and Govendjee 1985). Since these charges arrive after each light flash, it was suggested that Cl^- binding occurred after each S-state transition. The release of Cl^- was associated with the release of a positive charge, i.e., a proton (H^+). Since there is no H^+ release during $S_1 \longrightarrow S_2$ transition, the unbinding of Cl^- was suggested not to occur after this transition. Thus, the absence of Cl^- could specifically affect the S_2 created. In this hypothesis, the function of Cl^- was to bind to the system when a positive charge arrived on the water oxidation complex, stabilizing the system, and to lead H^+s away from the system by subsequent unbinding from the system. The reversible binding of Cl^- (high exchange rate, mentioned earlier) and the low binding energy ($\sim$12 kJ/mole) (Baianu et al. 1984) supported this picture. In a detailed elaboration on a possible mechanism of Cl^- action, Coleman and Govindjee (1985) have proposed how Cl^- might activate the base catalysis of H^+ removal from water. Here, a negatively charged group B^- functions to extract H^+s from water, whereas Mn functions to extract electrons from H_2O and Cl^- functions to bind transiently (and thus, reversibly) to positively charged group N^+ that raises the pKa of B^-. The N^+ may represent acidic amino acids. The proposed juxtaposition of a positively and

negatively charged group in the anion binding domain is consistent with the pH dependence of Cl^- release and rebinding, at least in a water oxidizing complex carrying all extrinsic polypeptides (Homann 1988a). As Cl^- binds to N^+, the pKa of the nearby B^- changes, and this increases its affinity to catalytic H_2O protons at the Mn-site making it easier to "pull" H^+s out of water, as Mn "pulls" electrons from H_2O. Both the H^+s and Cl^- leave the catalytic site at the same time. H^+s may then be conducted through the suggested 33 kDa Cl^--binding extrinsic polypeptide to the outside, i.e., into the lumen. Coleman and Govindjee (1987b) consider one possible Cl^- binding domain to be His 332 and 337 on D1 and His 337 on D2; if this is so, the Mn binding domains could be on the acidic amino acids: D308, E329, D319, E333, and D342 on the carbolytic containing loop of D_1 and E313, D334, E324, E338 and E345 on D_2. Whether and how such a binding and mechanisms of action can accomodate the structural effects of Cl^-, e.g., with respect to the binding of the small extrinsic polypeptides, remains an unsolved problem.

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