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**The mechanism of chloride activation of oxygen evolution in  
spinach photosystem II**

**Coleman, William Joseph, Ph.D.**

**University of Illinois at Urbana-Champaign, 1987**

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THE MECHANISM OF CHLORIDE  
ACTIVATION OF OXYGEN EVOLUTION  
IN SPINACH PHOTOSYSTEM II

BY

WILLIAM JOSEPH COLEMAN

B.A., University of Pennsylvania, 1979

THESIS

Submitted in partial fulfillment of the requirements  
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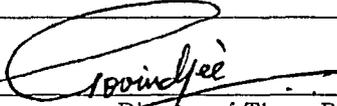
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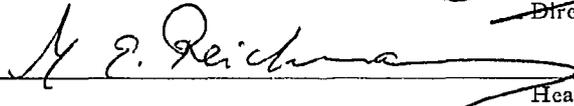
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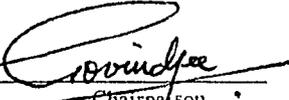
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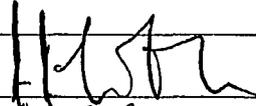
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To my parents

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The human body is a magical vessel, but its life is linked with an element it cannot produce. Only the green plant knows the secret of transforming the light that comes to us across the far reaches of space. There is no better illustration of the intricacy of man's relationship with other living things.

--Loren Eiseley,

Essay entitled "The Hidden Teacher"

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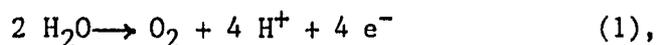
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# I. CHLORIDE ACTIVATION OF OXYGEN EVOLUTION IN PHOTOSYSTEM II: A MECHANISTIC PERSPECTIVE

## A. Introduction

A number of enzymes and proteins that catalyze acid-base reactions or that are involved in proton uptake and release (see section H) require chloride as a cofactor. The oxygen-evolving complex (OEC) of Photosystem II (PS II) is such an enzyme (for reviews, see Critchley, 1985; Govindjee et al., 1985; Renger and Govindjee, 1985; Homann and Inoue, 1986; Homann, 1987). The light-driven catalytic reaction has the following stoichiometry:

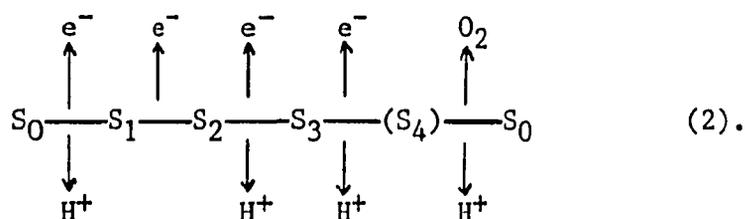


with an average  $E^0$  (mid-point redox potential at pH 7.0) of +0.81v per electron.

Considerable effort has been directed toward understanding the electron transfer reactions on the donor side of PS II. Electrons removed from water by the transiently oxidized manganese-complex are transferred to an intermediate one-electron carrier (Z) which is the immediate donor to P680, the reaction center chlorophyll (for a review, see Govindjee et al., 1985; Renger, 1987). During the initial stages of the light-induced charge separation, P680 is oxidized and Pheophytin (Pheo) is reduced. This event is followed by the reduction of the acceptor-side quinones ( $Q_A$  and  $Q_B$ ; Vermaas and Govindjee, 1981; Crofts and Wraight, 1983).

Coupling of the four-electron oxidation of water to the single-

electron turnover of the central reaction center components (Z-P680-Pheo-Q<sub>A</sub>) requires that the OEC be able to store four oxidizing equivalents at the Mn active site (see review by Wydrzynski, 1982) during each catalytic cycle. The complete cycle for water oxidation has been represented as a series of several transient states ("S-states") of the OEC (Kok et al., 1970; Forbush et al., 1971; Fowler, 1977; Saphon and Crofts, 1977):



It is clear from this reaction mechanism that removal of one of the products (H<sup>+</sup>) is probably essential to the advancement of certain S-states, if electron removal for that step is rate-limited by proton abstraction (Forster and Junge, 1985) or back-reaction. Theoretical models for the overall mechanism of O<sub>2</sub>-evolution and S-state turnover have recognized the importance of having an efficient proton-withdrawing mechanism (Kusonoki et al., 1980; Krishtalik, 1986).

Experimental evidence indicates that chloride (Cl<sup>-</sup>) ions are required for both steady-state O<sub>2</sub>-evolution (see, e.g., Kelley and Izawa, 1978) and for the advancement of particular S-states (Itoh et al., 1984; Theg et al., 1984). In addition, Cl<sup>-</sup> appears to be intimately involved in the pH-dependent activation of the enzyme (Gorham and Clendenning, 1952; Homann et al., 1983; Critchley, 1985). It is reasonable to expect that definable similarities might exist between the mechanism of Cl<sup>-</sup> action in the OEC and in other chloride-activated

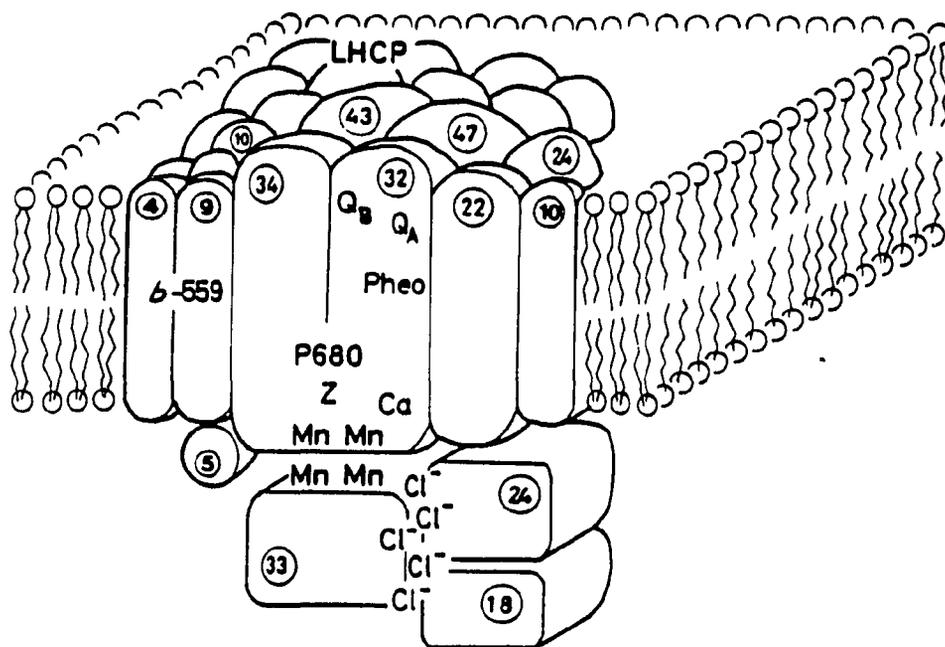
enzymes and proteins. Although the OEC is clearly a unique enzyme in terms of its overall function, particular details of its structure and mechanism that resemble other enzymes can be examined by comparative enzymology.

Experimentally, the current challenge in this area of photosynthesis research is to understand the mechanism of  $\text{Cl}^-$  binding and to relate this binding to the subunit structure of the enzyme and the kinetics of  $\text{O}_2$ -evolution. This review will focus on relevant similarities between the structure and catalytic mechanism of the OEC and other chloride-requiring enzymes, for the purpose of exploring potential new areas of investigation--even beyond the scope of this thesis.

## **B. Structure of the OEC**

The polypeptide structure of the OEC (Fig. 1) has been extensively reviewed (see, e.g., Ghanotakis and Yocum, 1985; Homann, 1987). From studies of inverted thylakoid vesicles, the complex is known to lie on the inner side of the thylakoid membrane (Åkerlund and Jansson, 1981; Yamamoto et al., 1981; Åkerlund et al., 1982; see also Blankenship and Sauer, 1974). An intrinsic polypeptide of  $M_r=34$  kD (known as "D2"; Chua and Gillham, 1977; Rochaix et al., 1984) appears to have a central role in both Mn binding (Metz and Bishop, 1980; Bishop, 1983), which is required for catalysis, and in the generation of a flash-induced EPR signal that is characteristic of the  $\text{S}_2$  state (Dismukes and Siderer, 1981; Miller et al., 1987; Styring et al., 1987). In association with another intrinsic membrane protein ( $\text{D}_1$ ,  $M_r=32$  kD), the D2 protein has been suggested to constitute part of the core of the PS II reaction center (Michel and Deisenhofer, 1985; Trebst and Draber, 1986; Nanba and

Figure 1. A model for the structure and organization of the oxygen-evolving PS II complex. P680 is the reaction center Chl, quinones  $Q_A$  and  $Q_B$  are the primary and secondary electron acceptors, and Z is the primary electron donor to P680. LHCP is the light-harvesting Chl protein. The PS II core complex contains proteins with MW 47 and 43 kD (Chl binding proteins), 34 and 32 kD (D2 and D1, respectively), and 9 and 4 kD (cyt  $b_{559}$ ). The extrinsic polypeptides involved in  $O_2$ -evolution have MW 33, 24, and 18 kD. Other proteins with MW 24, 22, 10 (2 proteins), and 5 kD have an unknown function. (After Murata and Miyao (1987).)



Satoh, 1987; Okamura et al., 1987).

The OEC also contains several extrinsic polypeptides. Some of these, having  $M_r < 10$  kD (Ljungberg et al., 1986), have only recently been revealed. The three largest extrinsic polypeptides ( $M_r=18, 24,$  and 33 kD) have been actively studied in order to determine their precise roles in  $O_2$ -evolution. One possible role for these proteins involves the binding of  $Ca^{2+}$ , which is required for  $O_2$ -evolution (Ghanotakis and Yocum, 1986). The presence of the 33 kD polypeptide is necessary for the other two polypeptides to bind to the membrane (Murata et al., 1983).

Selective removal of these extrinsic polypeptides can be achieved by washing PS II-enriched membrane fragments (known as PS II particles or membranes; Berthold et al., 1981; Kuwabara and Murata, 1982) or inside-out thylakoid vesicles, with high concentrations of various salts or denaturants (Åkerlund and Jansson, 1981; Åkerlund et al., 1982). Washing with 1.0 M NaCl removes predominantly the 18 kD and 24 kD polypeptides, as well as some of the bound  $Ca^{2+}$ , without affecting the functional Mn (Murata et al., 1983; Cammarata and Cheniae, 1987). Washing with 1.0 M  $CaCl_2$  removes all three polypeptides (Ono and Inoue, 1983). Removal of the 33 kD polypeptide (along with the 18 kD and 24 kD polypeptides) accelerates the loss of Mn from the membrane unless a high concentration of  $Cl^-$  (about 200 mM) is present in the medium (Miyao and Murata, 1984b). Washing with 0.8 M Tris at pH 8.0 removes all of the extrinsic polypeptides (Yamamoto et al., 1981) as well as the functional Mn (Murata et al., 1983).

Two other inhibitory treatments deserve mention here. Incubation with millimolar concentrations of  $NH_2OH$ , which removes a substantial amount of functional Mn, does not appear to affect the binding of the

extrinsic polypeptides, although  $O_2$ -evolution is inactivated (Murata et al, 1983). Mild heating (3-5 min, 35-50°C) also removes Mn and inactivates  $O_2$ -evolution, but its effect on polypeptide removal depends on the temperature (Nash et al., 1985).

The 33 kD polypeptide appears to be the most essential extrinsic subunit (see Homann, 1987). Although its amino acid sequence shows partial homology with Mn-superoxide dismutase from E. coli (Oh-oka et al., 1986), it is not clear whether this protein binds functional Mn in situ (Hunziker et al., 1987; Miller et al., 1987). Two regions of the primary sequence also show partial homology with the  $Ca^{2+}$  binding sites in calmodulin and troponin C (Coleman et al., 1987). The structural and functional linkage between  $Ca^{2+}$  and  $Cl^-$  binding sites is currently an active area of research (Homann and Inoue, 1986) and will be discussed in more detail in sections C and E.

### **C. Number and Location of $Cl^-$ Binding Sites.**

Up to now, attempts to determine the number of  $Cl^-$  binding sites in spinach have relied on measurements of radioactive  $^{36}Cl$  binding (Theg and Homann, 1982) and on  $O_2$ -evolution in a modulated electrode (Sinclair, 1984). Both measurements suggest that more than one  $Cl^-$  binding site is involved in  $O_2$ -evolution. Studies of the location of these binding sites have generally involved selective removal of the extrinsic polypeptides. Our knowledge of this aspect of the system is therefore limited to the polypeptide level.

An estimate of the effect of polypeptide removal on the apparent affinity of the OEC for  $Cl^-$  can be determined by measuring the steady-state rate of  $O_2$ -evolution as a function of the concentration of  $Cl^-$

added to  $\text{Cl}^-$  depleted membranes. Miyao and Murata (1985) have shown that PS II membranes which lack all of the extrinsic polypeptides reach a low  $V_{\max}$  (about  $30 \mu\text{mol O}_2 (\text{mg Chl})^{-1}\text{hr}^{-1}$ ) at about 150 mM  $\text{Cl}^-$ . When the 33 kD polypeptide is present,  $V_{\max}$  increases to about  $120 \mu\text{mol O}_2 (\text{mg Chl})^{-1}\text{hr}^{-1}$  at 30 mM  $\text{Cl}^-$ . In untreated PS II membranes,  $V_{\max}$  is greater than  $300 \mu\text{mol O}_2 (\text{mg Chl})^{-1}\text{hr}^{-1}$  at about 10 mM  $\text{Cl}^-$ . Similar results have been reported by Imaoka et al. (1986). The greatest contribution of the extrinsic polypeptides to the overall rate is in the range of low  $[\text{Cl}^-]$ , particularly for the case of the 18 kD and 24 kD polypeptides. These two subunits exhibit the greatest effect at low  $[\text{Cl}^-]$  when they are present together (Miyao and Murata, 1985). Although part of this synergism reflects the fact that the 24 kD polypeptide (which binds to the 33 kD polypeptide) is needed to provide a binding site for the 18 kD polypeptide (Miyao and Murata, 1983), it is nevertheless clear from these depletion/reconstitution studies that the 18 kD polypeptide increases the apparent affinity of the OEC for  $\text{Cl}^-$  in the region of 0.1-1.0 mM  $\text{Cl}^-$ ; that is, in the range of the apparent dissociation constant for activation by  $\text{Cl}^-$  ( $K_A'$ ). Its role in  $\text{Cl}^-$  stimulation of  $\text{O}_2$ -evolution is therefore not trivial.

Determining the true requirement for each of these polypeptides has been complicated, however, by the observation that various non-physiological concentrations of  $\text{Ca}^{2+}$  can apparently replace their function to a limited extent (Ghanotakis et al., 1984a; Imaoka et al., 1984; Nakatani, 1984). For example, in PS II membranes depleted of the extrinsic polypeptides, addition of 100 mM NaCl alone restores about 20% of the original activity. When this is supplemented with an additional 10 mM  $\text{CaCl}_2$ , nearly 40% of the original activity is restored (Kuwabara et al., 1985; see also Ono and Inoue, 1984). In PS II membranes

depleted of only the 18 kD and 24 kD polypeptides, addition of 20 mM NaCl alone restores only 26% of the original activity, whereas 10 mM CaCl<sub>2</sub> restores 80% of the original activity (Ghanotakis et al., 1985). Even when the 18 kD and 24 kD polypeptides are added back after depletion, the presence of Ca<sup>2+</sup> is still a crucial factor, because if these two subunits are added back after having been dialyzed against EGTA to remove Ca<sup>2+</sup>, the two polypeptides bind to the membrane but do not stimulate O<sub>2</sub>-evolution (Ghanotakis et al., 1984b). Nevertheless, these two polypeptides evidently enhance the binding of Ca<sup>2+</sup>, reducing the apparent dissociation constant to the more physiologically relevant micromolar level (Ghanotakis et al., 1984b).

What is clear from these studies is that no single polypeptide is likely to encompass all of the Cl<sup>-</sup>/Ca<sup>2+</sup> functions. Instead, it appears that there are multiple binding sites for Cl<sup>-</sup> and Ca<sup>2+</sup> (see, e.g., Boussac et al., 1985; Cammarata and Cheniae, 1987; Katoh et al., 1987) on different polypeptides. If the binding of these ions involves interactions between various subunits, then removal of any part of an interacting pair or group will significantly decrease the apparent affinity of the remaining subunits for Cl<sup>-</sup> or Ca<sup>2+</sup>. This property of the system would tend to create a "layering" effect; that is, removal of successive layers of extrinsic polypeptides (18, 24 kD → 33 kD → D1, D2) would progressively reduce the apparent OEC affinity for these activators (and the maximum catalytic rate) without completely abolishing activity. Each layer would appear to contribute a percentage to the overall efficiency of O<sub>2</sub>-evolution. The innermost layer, if it directly involves the intrinsic polypeptides, cannot be physically removed without damaging the Mn active site and the reaction center

structure. In this respect, both  $\text{Cl}^-$  and  $\text{Ca}^{2+}$  appear to function over an extended region.

Nevertheless, the extrinsic 33kD polypeptide seems to be the linchpin for the assembly of the  $\text{Cl}^-/\text{Ca}^{2+}$  activatable complex. In addition to stabilizing at least half of the catalytic Mn associated with the intrinsic proteins (Kuwabara et al., 1985; Imaoka et al., 1986), this polypeptide appears to bind both the 24 kD polypeptide (Andersson et al., 1984; Ljungberg et al., 1984) and other PS II core polypeptides (Bowlby and Frasch, 1986; Liveanu et al., 1986).

#### **D. Relationship of $\text{Cl}^-$ Binding to Mn Binding**

In several published models for  $\text{Cl}^-$  activation of the OEC,  $\text{Cl}^-$  functions as a Mn ligand in the active site (Sandusky and Yocum, 1983, 1984; Critchley and Sargeson, 1984). Experiments from various laboratories have indicated that  $\text{Cl}^-$  depletion affects both the EPR properties of the Mn center (Damoder et al., 1986) and its accessibility to inhibitors, such as  $\text{NH}_3$  (Sandusky and Yocum, 1983, 1984, 1986). Damoder et al. (1986) found that the depletion of  $\text{Cl}^-$  from PS II membranes significantly reduces the amplitude of the flash-induced multi-line EPR signal attributed to the  $\text{S}_2$  state. Their analysis of the effect of  $\text{F}^-$  competition on  $\text{Cl}^-$  enhancement of the  $\text{S}_2$  EPR signal yielded two dissociation constants for  $\text{Cl}^-$ , which they suggest reflects two  $\text{Cl}^-$  binding sites on the donor side of PS II. Although they raise the possibility that  $\text{Cl}^-$  binding to Mn could be involved in shifting the redox potential for  $\text{Mn}^{4+} \rightarrow \text{Mn}^{3+}$  in aqueous medium (see also Kambara and Govindjee, 1985), they concede that there is little evidence available to support the idea of direct binding of  $\text{Cl}^-$  to Mn.

Using a different approach, Sandusky and Yocum (1983, 1984, 1986)

studied the competition between  $\text{NH}_3$  and  $\text{Cl}^-$  by measuring steady-state  $\text{O}_2$ -evolution in  $\text{Cl}^-$  depleted PS II membranes. These authors concluded that  $\text{NH}_3$  inhibits  $\text{Cl}^-$  activation of  $\text{O}_2$ -evolution by direct competition at the  $\text{Cl}^-$  binding site. Since amines have been shown to interfere with the reactions at the catalytic Mn (Velthuys, 1975; also see Beck and Brudvig, 1986, 1987; Andreasson and Hansson, 1987), it was proposed by Sandusky and Yocum (1983; and later enlarged upon by Critchley and Sargeson, 1984) that  $\text{Cl}^-$  might act as a bridging ligand between the Mn atoms, and that  $\text{NH}_3$  inhibits the system by displacing  $\text{Cl}^-$ . Homann (1986), however, has reported that the protective effect of  $\text{Cl}^-$  in the presence of amines and other Lewis acids is indirect, perhaps involving changes in the tertiary and quaternary structure of the polypeptides.

Recent EXAFS (Extended X-ray Absorption Fine Structure) and EPR measurements also indicate that chloride ions and other halides are not inner-sphere ligands to the Mn that is observed by these techniques. Using EXAFS, Yachandra et al. (1986a) have reported that PS II Mn in the  $\text{S}_1$ -state does not show any involvement of  $\text{Cl}^-$  or ( $\text{Br}^-$ ) in the first coordination sphere. Likewise, by observing the EPR fine structure of Mn in the  $\text{S}_2$ -state, Yachandra et al. (1986b) found no difference between  $\text{Cl}^-$  and  $\text{Br}^-$ , which suggests that halides do not interact directly with Mn in this state. The EPR signal amplitude increased hyperbolically with the  $\text{Cl}^-$  concentration, however, indicating that a site to which  $\text{Cl}^-$  binds exerts an indirect effect on the EPR-active Mn. A possible explanation for the discrepancies in all of these results might be that although Mn and  $\text{Cl}^-$  bind to different sites, amines are capable of binding to (and inhibiting) both sites.

## E. The Kinetics of $\text{Cl}^-$ Activation

### 1. Effect on the S-states

The effect of  $\text{Cl}^-$  depletion on the advancement, stability, and deactivation of the S-states has been examined only during the last several years. Most reports indicate that the higher S-state transitions are primarily affected, although a few have suggested that  $\text{S}_0 \rightarrow \text{S}_1$  and  $\text{S}_1 \rightarrow \text{S}_2$  might also be involved (Damoder et al., 1986; Homann et al., 1986). The first studies of flash- $\text{O}_2$  yields and delayed light emission by Muallem et al. (1980) and Muallem and Boszormenyi (1981) demonstrated that removal of  $\text{Cl}^-$  stabilizes the  $\text{S}_2$  and  $\text{S}_3$  states by slowing down the rate of their deactivation. Itoh et al. (1984), monitoring Chl fluorescence yield changes, and Theg et al. (1984), monitoring fluorescence yield and delayed light emission, confirmed that  $\text{Cl}^-$  depleted thylakoids were capable of donating two electrons to the oxidized PS II reaction center (i.e., they were capable of generating the  $\text{S}_2$  state). The latter group, however, questioned the earlier conclusion that the rate of deactivation is slowed by  $\text{Cl}^-$  depletion, since they observed a higher initial luminescence level and an accelerated luminescence decay in depleted samples in the 50 ms to 1 s interval after illumination.

Other measurements of S-state turnover using thermoluminescence (Homann et al., 1986) and a modulated  $\text{O}_2$ -electrode (Sinclair, 1984) indicate that the  $\text{S}_3 \rightarrow (\text{S}_4) \rightarrow \text{S}_0$  reaction is effectively blocked by  $\text{Cl}^-$  depletion. Homann et al. (1986) suggest that earlier fluorescence measurements could not detect the very slow  $\text{S}_2 \rightarrow \text{S}_3$  transition in  $\text{Cl}^-$  depleted samples because of instrumental limitations. As Itoh et al. (1984) noted, the steady-state electron transfer from the OEC to  $\text{Z}^+$

changes from having a single half-time of 450  $\mu$ s in  $\text{Cl}^-$  sufficient thylakoids to having biphasic kinetics with a fast component of 120 ms and a slower component of several seconds in the  $\text{Cl}^-$  depleted case. Thus, Homann et al. (1986) argue that  $\text{Z}^+$  was not capable of being re-reduced within the 60 ms interval between flashes, and that the  $\text{S}_3$  state was therefore never observed (see also Inoue, 1987). Other thermoluminescence measurements, however, support the idea that the  $\text{S}_2 \rightarrow \text{S}_3$  transition is blocked by  $\text{Cl}^-$  depletion (Rozsa and Demeter, 1987).

On the basis of EPR evidence, Ono et al. (1986,1987) have argued that  $\text{Cl}^-$  depleted PS II membranes cannot advance beyond the  $\text{S}_2$  state, and that the  $\text{S}_2$  state produced after two flashes is EPR silent (see also Yachandra et al., 1986). This modified  $\text{S}_2$  state can be re-converted to an EPR-observable  $\text{S}_2$  state (i.e., one having a low-temperature multi-line signal at  $g=2$ ) simply by restoring  $\text{Cl}^-$  to the suspension medium. The light-induced  $g=4.1$  signal (also due to Mn) is not, however, affected by  $\text{Cl}^-$  depletion.

The light-induced  $\text{S}_2$  EPR signal also disappears when all of the extrinsic polypeptides have been removed, but is restored by the addition of 200 mM NaCl (Styring et al., 1987) or 200 mM NaCl plus 15 mM  $\text{CaCl}_2$  (Miller et al., 1987). Vass et al. (1987) have shown that although depletion of  $\text{Cl}^-$  and depletion of the 33 kD and other extrinsic polypeptides reversibly block the advancement of the S-states, the two treatments have different effects on the stability of the  $\text{S}_2\text{Q}_\text{A}^-$  and  $\text{S}_2\text{Q}_\text{B}^-$  states, as determined by thermoluminescence.

Only one study of  $\text{Cl}^-$  binding to the S-states has been done by  $^{35}\text{Cl}$ -NMR: Preston and Pace (1985) observed  $\text{Cl}^-$  binding to  $\text{Cl}^-$  depleted

PS II membranes from a halophyte (mangrove), and determined that high-affinity  $\text{Cl}^-$  binding involves primarily the  $\text{S}_2$  and  $\text{S}_3$  states.

## 2. Effect on steady-state electron transport

Warburg and Luttgens (1944) were the first to demonstrate the " $\text{Cl}^-$  effect" on  $\text{O}_2$ -evolution in dialyzed chloroplasts. Many years later, a series of experiments by Bove et al. (1963), Hind et al. (1969), Izawa et al. (1969), and Heath and Hind (1969) eventually pinpointed the site of  $\text{Cl}^-$  action on the donor side of PS II in glycophytes. The same site for the  $\text{Cl}^-$  effect was established in halophytes by Critchley et al. (1982). Hind et al. observed that  $\text{Cl}^-$  depletion significantly reduces the quantum efficiency of uncoupled electron transport. Heath and Hind reached a similar conclusion based on measurements of fluorescence yield. Izawa et al. showed, in addition, that  $\text{Cl}^-$  depletion reduces the maximum attainable rate of electron transport at a given light intensity.

In  $\text{Cl}^-$  depleted PS II membranes lacking the 18 kD and 24 kD polypeptides, addition of  $\text{Cl}^-$  improves the quantum efficiency, but does not enhance the maximum attainable rate of  $\text{O}_2$ -evolution (Homann and Inoue, 1986). Addition of  $\text{Ca}^{2+}$  in the presence of sufficient  $\text{Cl}^-$  was found to raise the quantum efficiency much more dramatically than it raised the maximum rate of  $\text{O}_2$ -evolution. Other studies, however, demonstrated a large effect of  $\text{Ca}^{2+}$  on the maximum rate of  $\text{O}_2$ -evolution in such preparations (see sections B and C).

Kelley and Izawa (1978) used uncoupled,  $\text{Cl}^-$  depleted thylakoids to obtain a kinetic estimate of the apparent activator dissociation constant ( $K_A'$ ) for  $\text{Cl}^-$  of approximately 0.9 mM (see also Baianu et al., 1984). This value is probably somewhat higher than the actual value due to the presence of 2 mg  $\text{ml}^{-1}$  bovine serum albumin (a  $\text{Cl}^-$  binding

protein; see section H) in the suspension. It is also relevant to note that in their experiment (and in others published since then), there is a measurable rate of  $O_2$ -evolution in the absence of any added  $Cl^-$  (see Kelley and Izawa, fig. 5). For the purposes of constructing double-reciprocal (Lineweaver-Burk) plots, this "endogenous" rate introduces a significant complication in estimating  $K_A'$  and other parameters, and should not be overlooked (see, e.g., Reiner, 1969). In this respect, the key question of whether  $Cl^-$  is an essential or non-essential activator (Segel, 1975) has not been thoroughly addressed.

#### **F. The $Cl^-$ Binding Mechanism**

A complete understanding of the mechanism of  $Cl^-$  activation of the OEC cannot be achieved by studying the  $O_2$ -evolution kinetics alone. A thorough investigation of the binding properties of  $Cl^-$  in this system will also be needed. In general, we would like to know the number and location of the  $Cl^-$  binding sites and the degree to which different sites might interact. More specifically, we would like to know: a) the nature of the exchange between bound and free  $Cl^-$ ; b) the affinity/binding energy; c) the relationship between anion size (or charge) and the ability of that anion to bind effectively and stimulate  $O_2$ -evolution; and d) the  $pK_a$ 's of the groups involved in binding and the effect of  $Cl^-$  on these  $pK_a$ 's.

A useful way to approach these questions is by using  $^{35}Cl$ -NMR. The initial  $^{35}Cl$ -NMR studies of  $Cl^-$  binding to  $Cl^-$  depleted thylakoids were limited by low instrumental sensitivity, and thus thylakoids from various halophytes were used, since they have a very high  $Cl^-$  requirement for  $O_2$ -evolution (see Critchley et al., 1982; Baianu et al., 1984). Baianu et al. found that the exchange rate for  $Cl^-$  between bound

and free environments is rapid ( $>10^4 \text{ s}^{-1}$ ), and that the observed increases in  $^{35}\text{Cl}$ -NMR linewidth upon  $\text{Cl}^-$  binding reflect contributions from quadrupolar relaxation. By measuring the  $^{35}\text{Cl}$ -NMR linewidth as a function of the concentration of added  $\text{Cl}^-$ , it is possible to construct a  $\text{Cl}^-$  binding curve, which can be used to calculate the  $K_A$  for  $\text{Cl}^-$  (see Baianu et al., 1984; reviewed in Coleman and Govindjee, 1987). Values obtained for  $K_A$  were 0.1 M and 0.14 M at  $5^\circ\text{C}$  and  $25^\circ\text{C}$ , respectively (after transformation from binding constants to dissociation constants). From these two constants it is also possible to calculate a binding energy of  $3 \text{ kcal mol}^{-1}$  for this system, which indicates weak ionic binding. This is consistent with the known reversibility of  $\text{Cl}^-$  association with the membrane.

Critchley et al. (1982) also analyzed the correlation between the relative effectiveness of various anions in stimulating  $\text{O}_2$ -evolution (which decreases in the order  $\text{Cl}^- > \text{Br}^- > \text{NO}_3^- > \text{I}^-$ ; Hind et al., 1969; Kelley and Izawa, 1978) and their relative sizes and ionic field strengths. Critchley et al. concluded that ionic field effects and steric factors control the binding of anions, such that the anion must have a certain optimum charge and size in order to activate the enzyme. In PS II membranes, Homann (1985) and Homann and Inoue (1986) have reported a similar result. Homann found that anions such as  $\text{NO}_3^-$  have higher apparent dissociation constants than  $\text{Cl}^-$ , and are lost from the membrane at a faster rate. Homann and Inoue found, however, that removal of the 18 kD and 24 kD polypeptides greatly diminishes the effectiveness of other anions relative to  $\text{Cl}^-$ , even in the presence of adequate  $\text{Ca}^{2+}$ . An anion dependence of the flash-induced  $\text{S}_2$  EPR signal has also been reported (Damoder et al., 1986).

The pH dependence of  $\text{Cl}^-$  activation of  $\text{O}_2$ -evolution in thylakoids

and PS II membranes has been examined numerous times since the early 1950's. Most of these studies have concerned the effect of  $\text{Cl}^-$  depletion and re-addition on the activity vs. pH curves for  $\text{O}_2$ -evolution. Gorham and Clendenning (1952) made the crucial observation that the addition of  $\text{Cl}^-$  to  $\text{Cl}^-$  depleted thylakoids both raised the maximum rate of electron transport and shifted the pH optimum in their system from about 6.4 to about 6.8 at  $20^\circ\text{C}$ . The net effect of this displacement is that the rate of  $\text{O}_2$ -evolution is greatly enhanced at alkaline pH. Since then, this effect has been confirmed in other systems (see, e.g., Critchley, 1985).

Massey (1953) has constructed a detailed mechanistic model to explain how anion binding to a positive charge in the active site of an enzyme might be capable of inducing such a shift in the velocity-pH optimum. He has proposed that two catalytically active residues exist in such enzymes, and that at the pH optimum, one is protonated and the other is unprotonated. When the anion combines with a third positively-charged residue, the electrostatic effect of its (+)charge is screened, thereby raising the  $\text{pK}_a$ 's of the two catalytic residues (see also Matthew and Richards, 1982). The net effect is always an alkaline shift in the pH optimum for enzyme activity.

Critchley et al. (1982) and Baianu et al. (1984) have used  $^{35}\text{Cl}$ -NMR to measure directly the pH dependence of  $\text{Cl}^-$  binding in thylakoids from halophytes. Critchley et al. (1982) found that the excess  $^{35}\text{Cl}$ -NMR linewidth, which reflects the fraction of  $\text{Cl}^-$  bound to the membrane, peaks at pH 7.2 in thylakoids from Avicennia germinans, and decreases on either side of this optimum. The pH dependence for  $\text{O}_2$ -evolution showed a parallel trend. The activity vs. pH curve for these thylakoids also

shows an alkaline shift as the  $[Cl^-]$  is increased (Critchley, 1983). In the latter case, Critchley suggested that the steepness of the curve on the alkaline side indicates ionization of as many as five basic groups. However, since there is the possibility that treatment at alkaline pH causes irreversible damage to the  $Cl^-$  binding sites, additional tests of the pH stability of these preparations and spinach preparations are needed (see, e.g., Segel, 1975).

Other attempts have been made with preparations from spinach to characterize the  $pK_a$  of the group or groups involved in  $Cl^-$  binding, with the goal of determining the particular kind of amino acid involved (if the binding site is composed of amino acid residues). In spinach thylakoids, however, complete  $Cl^-$  depletion is difficult to achieve. Since  $Cl^-$  deficiency can be induced by high pH treatment, the depletion process often involves incubation at high pH (Kelley and Izawa, 1978; Theg and Homann, 1982; Theg et al., 1982; Izawa et al., 1983). Chloride depletion is further accelerated in thylakoids by treatments with uncouplers such as EDTA (Izawa et al., 1969) or protonophores (Theg and Homann, 1982; Theg et al., 1982) by competition with sulfate (Izawa et al., 1969) and by avoidance of illumination (Theg and Homann, 1982; Theg et al., 1982). The likely explanation for these diverse phenomena is that the bound  $Cl^-$  ions are tightly associated with a sequestered pool of protons (Dilley et al.; Theg and Homann, 1982; Theg et al., 1982). Treatments which deplete this slowly-equilibrating pool therefore accelerate  $Cl^-$  release. The  $pK_a$  for  $Cl^-$  release in thylakoids is greater than 8.0, indicating that amine derivatives (Homann et al., 1983), such as lysine or the terminal alpha-amino groups of the polypeptides, might be involved. In the case of lysine, such a  $pK_a$  would be anomalously low. This suggests that clusters of positively-

charged residues or the proximity of bound cations (such as  $\text{Ca}^{2+}$ ) might be involved in determining the observed  $\text{pK}_a$  (see section H). It is also interesting to note that addition of a small amount of  $\text{Mg}^{2+}$  shifts the apparent  $\text{pK}_a$  for  $\text{Cl}^-$  release to lower pH (Homann et al., 1983; Homann, 1985).

Thorough  $\text{Cl}^-$  depletion of PS II membranes requires more delicate procedures than in thylakoids. Since the OEC polypeptides are not enclosed within the lumen, they are more susceptible to detachment at high pH (Chapman and Barber, 1987). For this reason, pH- or anion-jump treatments are often employed (Homann, 1985; Itoh and Iwaki, 1986).

Homann (1985) measured the apparent dissociation constant for  $\text{Cl}^-$  as a function of pH in PS II membranes, and was able to show that  $\text{Cl}^-$  binding requires protonation of a group having a  $\text{pK}_a=6.0$ . This value seems to contradict the earlier observation in thylakoids that  $\text{Cl}^-$  release requires a  $\text{pH}>8$  (see discussion in Homann, 1985). A discrepancy also appears when comparing the rapid rate of  $\text{Cl}^-$  reactivation of the OEC with the relatively slow rate of  $\text{Cl}^-$  depletion (Homann, 1985). This apparent hysteresis may reflect slow conformational changes in the OEC (see Kurganov, 1982). This effect may also be related to the slow changes that have been observed in the  $\text{S}_2$ -state EPR signal in PS II membranes that have been dark-adapted for various periods of time before illumination (Beck et al., 1985).

#### **G. $\text{Cl}^-$ Effects on OEC Conformation**

It has long been known that mild heat treatment accelerates the dissociation of  $\text{Cl}^-$  from the OEC in  $\text{Cl}^-$  free medium (Hind et al., 1969). More recently, it was also found that  $\text{Cl}^-$  depletion accelerates the

thermal inactivation of  $O_2$ -evolution in thylakoids (Coleman et al., 1984). Re-addition of  $Cl^-$  to the medium immediately before heating partially protects against the loss of activity, whereas other anions, such as  $Br^-$ ,  $NO_3^-$ , and  $SO_4^{2-}$ , provide a lesser degree of protection (Coleman et al., 1984), in accordance with their ability to stimulate  $O_2$ -evolution in unheated membranes. Chloride also appears to protect against inactivation by 0.8 M Tris (Izawa et al., 1983). Protection by  $Cl^-$  against these two protein-denaturing treatments suggests that  $Cl^-$  interaction with a specific site or sites changes the energetics of unfolding and stabilizes the OEC against inactivation, by analogy with other ligand-enzyme systems (see, e.g., Zyk et al, 1969; Gill et al., 1985).

## **H. Comparisons with Other $Cl^-$ Binding Enzymes and Proteins**

### **1. Introduction**

As noted in section A,  $Cl^-$  is involved in the mechanisms of numerous enzymes and proteins that catalyze hydrolysis, protonation/deprotonation, and proton transport. The objective of this section is to analyze several of these proteins and identify the features they may have in common with the OEC, in order to shed more light on the mechanism by which  $Cl^-$  activates  $O_2$ -evolution.

Potential binding sites for  $Cl^-$  in proteins include the amino acid side chains of histidine, lysine and arginine, the terminal alpha-amino groups, and various metal cofactors. Interactions between various charged side chains and between bound metal ions and these groups may also strongly influence the affinity of the binding site for  $Cl^-$ . An additional factor is, of course, the state of protonation of the

positively-charged  $\text{Cl}^-$  binding groups, which depends on the charges present within the immediate environment of the binding site itself, and on any linkage which may exist between the binding site and other regions of the protein. A complete description of  $\text{Cl}^-$  binding to any given site is therefore sometimes complicated by a number of other considerations.

## 2. $\text{Cl}^-$ binding to metal sites

There are a number of  $\text{Cl}^-$  requiring metalloenzymes that have been studied in detail. Myeloperoxidase, for example, is a chlorine-containing enzyme that has two  $\text{Cl}^-$  binding sites. One of these sites, which is unaffected by pH, is believed to be the  $\text{Cl}^-$  binding site. Chloride binding to the second site is pH-dependent, such that the site must be protonated ( $\text{pK}_a \sim 4.5$ ) before  $\text{Cl}^-$  will bind. Chloride binding at this second site is competitive with  $\text{H}_2\text{O}_2$  binding (Andrews and Krinsky, 1982). Ikeda-Saito and Prince (1985) found that  $\text{Cl}^-$  lowers the  $E_m$  for the Fe(II)/Fe(III) redox transition from 143 mV to 21 mV at pH 4.3, but has very little effect at pH 7.9. Chloride binding also appears to reduce the rhombicity of the high-spin EPR signal. These authors interpret the interaction between  $\text{H}_2\text{O}_2$  and  $\text{Cl}^-$  as a competition between the two ligands for direct binding to the Fe at the sixth coordination position. They also suggest that although  $\text{Cl}^-$  displacement of the  $E_m$  is inhibited by high pH,  $\text{Cl}^-$  and  $\text{OH}^-$  do not compete directly (or at least not to the same enzyme conformation), since the enzyme appears to have a substantially higher affinity for  $\text{OH}^-$ . A similar conclusion was drawn earlier by Critchley et al. (1982) and Critchley (1983) regarding apparent competition between binding of  $\text{Cl}^-$  and  $\text{OH}^-$  to the OEC in thylakoids from halophytes.

Two  $\text{Cl}^-$  binding sites have also been proposed for a somewhat similar enzyme, chloroperoxidase (Lambeir and Dunford, 1983). One of these sites, which binds  $\text{Cl}^-$  only at low pH, inhibits compound I formation (a transient oxy-enzyme complex). Lambeir and Dunford attribute this inhibition to direct binding of  $\text{Cl}^-$  to the sixth coordination position of the heme iron because of the observed competition between  $\text{Cl}^-$  and  $\text{CN}^-$ , an anion which is known to occupy that site. Chloride is also a substrate, however, since it reacts with compound I to form a second complex known as the halogenating intermediate (Sono et al., 1986). Sono et al. argue that this second  $\text{Cl}^-$  binds covalently to compound I to form the halogenating intermediate, and does not ligate directly to the iron. They also suggest that the inhibitory  $\text{Cl}^-$  ion interacts primarily with a protonated amino acid residue in the vicinity of the active site.

In ferricytochrome c peroxidase,  $\text{Cl}^-$  can only bind to the sixth position of the heme iron at  $\text{pH} < 5.5$ , when a nearby residue is protonated (Hashimoto et al., 1986). Protonation of this residue is suggested to cause a structural change in the heme environment. This change involves movement of a key  $\text{H}_2\text{O}$  molecule in the sixth coordination position, such that  $\text{Cl}^-$  access to the Fe is no longer blocked.

In horseradish peroxidase,  $\text{Cl}^-$  (but not  $\text{NO}_3^-$ ) is suggested to bind directly to the sixth coordination position of the Fe (Araiso and Dunford, 1981). Nitrate is believed to bind instead to another nearby site on an amino acid residue whose protonation at low pH is required for binding.

It is interesting to compare the  $\text{Cl}^-$  binding properties of these peroxidases with those of the OEC. Although  $\text{Cl}^-$  is not involved as a

substrate in water oxidation, "hydrogen peroxide" has been proposed as a Mn-bound intermediate (see, e.g., Kamabara and Govindjee, 1985); it also appears to interact with the catalytic Mn in PS II membranes when the extrinsic polypeptides have been removed (Berg and Seibert, 1987). The secondary  $\text{Cl}^-$  binding site on the peroxidases, which probably does not involve the Fe, may have relevance to  $\text{Cl}^-$  activation of the OEC, since pH-dependent  $\text{Cl}^-$  binding to this site affects the redox and EPR properties of the enzyme, at least in the case of myeloperoxidase. Chloride binding to the OEC likewise affects the EPR properties of the  $\text{S}_2$  state (see section E1), and may also influence the redox state of the complex (Vass et al., 1987). The  $\text{pK}_a$ 's of groups involved in  $\text{Cl}^-$  binding are, however, very different in these two systems. It should be noted here that another heme-containing enzyme, cytochrome c oxidase, binds  $\text{Cl}^-$  specifically at the  $\text{cyt } a_3/\text{Cu}_B$  site, but only when both metal centers are oxidized (Blair et al., 1986). This binding alters the redox interaction between the metal centers. Cytochrome c oxidase also interacts with  $\text{Ca}^{2+}$  at the  $\text{cyt } a$  site (Saari et al., 1980).

Chloride binding to zinc-, cadmium-, and manganese-containing active sites has also been examined, although the metal coordination chemistry is not as well characterized as in the heme complexes. In alkaline phosphatase, which in its native form is a Zn-enzyme,  $^{35}\text{Cl}$ -NMR and  $^{113}\text{Cd}$ -NMR were used by Gettins and J.E. Coleman (1984) to study  $\text{Cl}^-$  binding to the  $^{113}\text{Cd}$ -substituted enzyme. For the phosphorylated, fully Cd-substituted enzyme, they concluded that  $\text{Cl}^-$  binds directly to one of the Cd ions by displacing  $\text{H}_2\text{O}$ . They also proposed that this displacement activates the enzyme by accelerating the dissociation of the phosphate. (This is in contrast to the inhibition observed in carbonic anhydrase, where  $\text{Cl}^-$  binding at or near the Zn atom displaces

the OH<sup>-</sup> (or H<sub>2</sub>O) ligand that is essential to the reaction (Ward and Cull, 1972).) In a phosphate-free, partially Cd-substituted form of the enzyme, Gettins and Coleman suggest that Cl<sup>-</sup> might be involved as a bridging ligand between the two metal centers, which are 3.9 Å apart. A similar placement for Cl<sup>-</sup> in the OEC has been proposed (see section D), but there is no extensive evidence for either model.

<sup>35</sup>Cl-NMR studies of another Zn-enzyme, carboxypeptidase A, led to the conclusion that the enzyme has two Cl<sup>-</sup> binding sites with fairly low affinities, and that the active site Zn atom is the primary Cl<sup>-</sup> binding site (Stephens et al., 1974; Stephens and Bryant, 1976). Since Cl<sup>-</sup> is actually an inhibitor of the enzyme, Williams and Auld (1986) examined the effect of Cl<sup>-</sup> on the enzyme kinetics, and found that Cl<sup>-</sup> is a partial competitive inhibitor of the substrate. Because the observed inhibitor dissociation constant (~50 mM) was much less than the lowest dissociation constant obtained by <sup>35</sup>Cl-NMR, they concluded that Cl<sup>-</sup> binds primarily to a substrate-binding amino acid residue in the active site (Arg 145) and not directly to the Zn.

Chloride binding to a third Zn enzyme, liver alcohol dehydrogenase, has also been suggested to occur at the catalytic metal ion in the horse enzyme (Maret and Zeppezauer, 1986), but the evidence is not yet definitive, since Cl<sup>-</sup> binding in the human enzyme can be affected by an Arg→His mutation at position 47 in the β<sub>2</sub> subunit (Bosron et al., 1986). These examples underscore the difficulty in unequivocally demonstrating exclusive Cl<sup>-</sup> binding to protein-linked metal ions, even in isolated, purified proteins.

Well-characterized examples of Cl<sup>-</sup> binding to Mn proteins or equivalent model compounds are relatively scarce. One unusual case

involves the Mn(II)-substituted form of alkaline phosphatase (Haffner et al., 1974). The low-temperature X-band EPR spectrum of this protein displays both a  $g=2$  signal and a prominent signal at  $g=4.3$ , which is characteristic of a  $d^5$  transition metal in a ligand environment with a large rhombic (tetrahedral) distortion. Similar low-field EPR signals have been observed in organo-metallic Mn complexes with  $Cl^-$  as a contributing ligand (Dowsing et al., 1969). This observation may have relevance to the OEC, where a low-field signal has been observed at  $g=4.1$ , but which is believed to involve only the higher valence states of Mn (dePaula et al., 1986; Aasa et al., 1987).

The conclusion that can be drawn from these comparisons with respect to the OEC is that reversible  $Cl^-$  binding to the catalytic Mn center would not be expected to stimulate water oxidation in those S-states where bound  $H_2O$  (and possibly  $H_2O_2$  and  $OH^-$ ) is involved, since  $Cl^-$  is capable of displacing  $H_2O$  from the metal coordination sphere. Oxidation of  $Cl^-$  might also be a problem. Stable coordination of  $Cl^-$  as a bridging ligand between Mn atoms is a theoretical possibility, but it is not supported by observations of the Mn ligand environment in the  $S_1$  and  $S_2$  states (see section D).

### 3. $Cl^-$ binding to basic amino acid residues

In addition to the enzymes described in the previous section, there are a number of transport proteins and enzymes that are activated by  $Cl^-$  binding to particular basic amino acid residues. This section will focus on seven well-known  $Cl^-$  binding proteins and compare them to the OEC with respect to the composition of their  $Cl^-$  binding sites, the  $Cl^-$  dependence of  $H^+$  binding, and any  $Cl^-$  mediated conformational changes.

The interaction of pancreatic ribonuclease A (RNase A) with various

anions has been extensively studied. The enzymatic activity has been shown to be sensitive to the ionic strength of the suspending medium. Kalnitsky et al. (1959) found that increasing the ionic strength (primarily by increasing the  $\text{Cl}^-$  concentration) at pH 5.3 activates the enzyme when the ionic strength is less than 0.2. At higher ionic strength, the enzyme is inhibited. This behavior is similar to what is observed in the OEC. Increasing the ionic strength above 0.1 also results in a downward shift in the pH optimum of RNase A, as well as a decrease in activity (Kalnitsky et al., 1959; Irie, 1965). These studies suggest that  $\text{Cl}^-$  binding to the protein alters the ionization state of critical residues in the active site.

On the basis of calculations from electrostatic models, the active site of RNase S (a catalytically active subfragment of RNase A) is predicted to have two partially occupied anion binding sites at pH 6.0 (Matthew and Richards, 1982). Earlier measurements of  $\text{H}^+$  and  $\text{Cl}^-$  binding had indicated that at pH 6.6, 0.5 moles of  $\text{Cl}^-$  ions are bound per mole of RNase A. When the basic residues are protonated at pH 4.5, 2.0 moles of  $\text{Cl}^-$  are bound (Saroff and Carroll, 1962). The positive electrostatic potential giving rise to the two anion binding sites is suggested to result from contributions by several basic amino acid residues in the vicinity, notably His 12, His 119, and Lys 41 (Matthew and Richard, 1982).

The clustering of charges within the active site of RNase A has a significant effect on the protonation state of these basic residues. The anomalously low  $\text{pK}_a$  of Lys 41, for example, has been attributed to the presence of nearby Arg 39 (Carty and Hirs, 1968). Jentoft et al. (1981) modified Lys 41 by  $^{13}\text{C}$ -methylation and observed its  $^{13}\text{C}$ -NMR

chemical shift as a function of pH. They concluded that a conformational change involving the movement of Lys 41 may be coupled to ionization of a particular histidyl residue, probably His 12. The apparent  $pK_a$ 's of His 12 and His 119 are also predicted to be pH dependent, as a consequence of the alteration in their electrostatic environment (Matthew and Richards, 1982). Chloride binding alters their protonation states still further. As the  $[Cl^-]$  is increased, the affinity of these two residues for  $H^+$  increases, consistent with their involvement in  $Cl^-$  binding (reviewed in Matthew and Richard, 1982). The degree to which  $Cl^-$  binding raises the  $pK_a$  of either His 12 or His 119 is predicted to depend on the distance from each His residue to each of the  $Cl^-$  binding sites. The protonation state of these two histidyl residues is extremely important to the observed enzyme activity, since His 12 is proposed to act as a proton acceptor and His 119 as a proton donor in the catalytic mechanism (Roberts et al., 1969).

Other effects on  $Cl^-$  binding at low pH have also been observed. Protonation of carboxyl groups by lowering the pH below 4.5 leads to the binding of additional  $Cl^-$  ions (Loeb and Saroff, 1964). Loeb and Saroff attributed this linkage between  $H^+$  and  $Cl^-$  at low pH to the existence of charge pairs within discrete clusters of acidic and basic residues. They proposed that neutralization of the acidic residues by  $H^+$  allows  $Cl^-$  to bind to the positively-charged residues.

The complex, pH-dependent  $Cl^-$  binding behavior in RNase A demonstrates the important contribution of electrostatic interactions between  $H^+$ ,  $Cl^-$ , and protonatable amino acid residues to the activity of the enzyme. Thus, depending on the ionization state required for catalytic activity,  $Cl^-$  may either activate or inhibit catalysis at an enzyme active site.

Another enzyme whose activity can be enhanced by the presence of  $\text{Cl}^-$  is the angiotensin converting enzyme (ACE), a Zn-containing hydrolase (exopeptidase) involved in blood-pressure regulation (for a review, see Cushman and Ondetti, 1980). In one study,  $\text{Cl}^-$  was found to raise  $V_{\text{max}}$  by over four-fold and the  $K_m$  by six-fold (Rohrbach et al., 1981). Overall, the enzyme requires about 100 mM  $\text{Cl}^-$  for maximal activity (Tsai and Peach, 1977) although the  $\text{Cl}^-$  requirement depends on the nature of the substrate. The role of  $\text{Cl}^-$  as either an essential or non-essential activator also depends on the particular substrate (Bunning and Riordan, 1983).

As with RNase A,  $\text{Cl}^-$  binding affects the height and position of the activity-pH optimum, shifting the curve to the alkaline side. Unlike RNase, however, ACE is still active at  $[\text{Cl}^-]$  above 0.1 M. The activity-pH curve for ACE is also considerably broadened at high  $[\text{Cl}^-]$ . The  $K_a'$  for  $\text{Cl}^-$  shows a pH-dependent increase between pH 6.0 and 9.0 (changing from 3.3 mM to 190 mM), and a critical lysine residue has been implicated in the binding (Shapiro and Riordan, 1983). The precise mechanism by which  $\text{Cl}^-$  activates the enzyme is not known, but may involve stabilization of a particular conformation at an allosteric site (Tsai and Peach, 1977; Shapiro and Riordan, 1984).

Pancreatic alpha-amylase (PAA) is a soluble enzyme that hydrolyzes oligosaccharides and requires both  $\text{Cl}^-$  and  $\text{Ca}^{2+}$  as cofactors (for a review, see Karn and Malacinski, 1978). The enzyme has an extremely high affinity for both of these ions. Calcium, which appears to stabilize the protein structure (Caldwell and Kung, 1953) binds at both a low-affinity and high-affinity site. The latter has a  $K_D$  of  $5 \times 10^{-12}$  M (Payan et al., 1980). Calcium alone does not activate the enzyme in

the absence of  $\text{Cl}^-$  (Caldwell and Kung, 1953). This has its parallel in the OEC.

Chloride seems to function as an allosteric activator, with a  $K_D=0.3$  mM at  $25^\circ\text{C}$  (Levitzki and Steer, 1974). It raises  $V_{\max}$  30-fold, but has no effect on the substrate  $K_M$ . Chloride binding appears to trigger a small, localized conformational change which suppresses the exchange of 26 protons and increases the apparent affinity of the enzyme for  $\text{Ca}^{2+}$  by 240-fold. In a later study, Lifshitz and Levitzki (1976) determined that there is one  $\text{Cl}^-$  binding site per molecule, a site which probably consists of a lysine  $\epsilon$ -amino group with  $\text{pK}_a=9.1$ . Calcium binding near this residue was suggested to explain this low  $\text{pK}_a$ . When this lysine is selectively modified,  $\text{Cl}^-$  binding is abolished, but substrate binding is not affected. The persistence of a low basal activity following this modification suggests that  $\text{Cl}^-$  is a non-essential activator (Lifshitz and Levitzki, 1976). Chloride binding also protects the enzyme against inactivation by heating at  $40^\circ\text{C}$  (Caldwell and Kung, 1953). This also has its parallel in the OEC (see Coleman et al., 1983; Nash et al., 1985).

As in the OEC, the function of  $\text{Cl}^-$  can be replaced by other monoanions, although they exhibit reduced effectiveness as activators (Levitzki and Steer, 1974). The dependence of the activity on the anionic size is similar to what has been observed in spinach thylakoids (see Critchley et al., 1982).

Unlike the OEC, the pH-activity curve for PAA is fairly broad (Wakim et al., 1969). Chloride binding does, however, shift the optimum to substantially higher pH, in addition to raising the relative velocity.

The behavior of  $\text{Cl}^-$  in PAA bears some similarity to the  $\text{Cl}^-/\text{OEC}$

system, especially with respect to the interdependence of  $\text{Cl}^-$  and  $\text{Ca}^{2+}$  and the suppression of  $\text{H}^+$  exchange by  $\text{Cl}^-$  binding. Other similarities include the low apparent dissociation constant for  $\text{Cl}^-$  (0.3 mM), the  $\text{pK}_a=9$  for the  $\text{Cl}^-$  binding site, and  $\text{Cl}^-$  induced resistance to heat inactivation. These features most closely parallel what is observed for  $\text{Cl}^-$  binding to the OEC in the presence of the extrinsic polypeptides (see sections B,C,E,F).

Albumins from human and bovine serum (HSA and BSA, respectively) are examples of the most basic class of  $\text{Cl}^-$  binding proteins, and have been exhaustively studied as model systems for ion binding to proteins (for a general review of structure and function, see Peters, 1985). In 1949, Scatchard and Black reported that the binding of  $\text{Cl}^-$  by HSA shifts its isoionic point (see Vesterberg, 1971) to higher pH, due to electrostatic interactions between the negatively-charged ion and positively-charged amino acid residues in the protein. Thus, the net effect of  $\text{Cl}^-$  binding is to protonate acidic groups on the protein and thereby raise the pH of the suspending medium (Louvrien and Sturtevant, 1971), a process which tends to preserve the electroneutrality of the macromolecule (Ifft and Vinograd, 1966). Chloride binding has been suggested to cause a conformational change in the protein that leads to a "tightening-up" of the structure (Louvrien and Sturtevant, 1971). The magnitude of the observed shift in the isoionic point also depends on the nature of the monoanion employed (Scatchard and Black, 1949).

The protein contains such a large number of  $\text{Cl}^-$  binding sites that they can only be grouped into classes (Scatchard and Yap, 1964). Class 1 has a  $K_D=1.4$  mM and consists of one group with a  $\text{pK}_a=8.0$  (possibly an alpha-amino). Class 2 has a  $K_D=16$  mM and consists of four groups with a

$pK_a=6.1$  (probably imidazole). Class 3 has a  $K_D=100$  mM and consists of 22 groups with high  $pK_a$ 's. Chloride binding to the lower affinity sites has been examined by  $^{35}\text{Cl}$ -NMR (Norne et al., 1975).

BSA/HSA appears to undergo several changes in conformation as the pH is lowered (Peters, 1985). When the pH is lowered below 5, additional  $\text{Cl}^-$  binding sites appear, possibly as a result of exposure of sequestered basic groups whose carboxylate partners have been protonated (Saroff, 1959). A conformational change in the neutral region appears to be affected by  $\text{Ca}^{2+}$  (Harmsen et al., 1971), which is known to bind to the protein (Edsall et al., 1950; Saroff and Lewis, 1963). This transition may involve a shift in the  $pK_a$ 's of imidazoles involved in internal salt bridges (Harmsen et al., 1971). Because of the thoroughness with which  $\text{Cl}^-/\text{H}^+$  uptake has been studied in this protein, serum albumin may provide a useful model for understanding this process in the OEC.

A thorough summary of the extensive literature on  $\text{Cl}^-$  binding to hemoglobin (Hb) is beyond the scope of this review, and therefore only the key elements will be discussed here. Chloride binding to this protein is intimately involved in a phenomenon known as the alkaline Bohr effect (for a review, see Perutz et al., 1980). The effect can be summarized as follows: As the pH falls from 9 to 6, the oxygen affinity of Hb drops, and the protein is converted from the oxy to the deoxy form. Simultaneously, however, the  $\text{H}^+$  affinity rises, enabling the protein to absorb  $\text{H}^+$  as  $\text{O}_2$  is released. Chloride appears to stabilize both  $\text{H}^+$  uptake and the oxy $\rightarrow$ deoxy conversion by binding preferentially to the deoxy form (Benesch et al., 1969).

Chloride binds to at least three residues in deoxy Hb: 1) Val 1 $\alpha$ , where it may be interlocked in a salt-bridge between the Val 1 $\alpha$   $\alpha$ -amino

group and the guanidinium group of Arg 141 $\alpha$  on the other  $\alpha$ chain (Arnone et al., 1976; O'Donnell et al., 1979). This binding shifts the  $pK_a$  of Val 1 $\alpha$  upward in deoxy Hb (O'Donnell et al., 1979). 2) Lys 82 $\beta$  (Arnone, 1972; Nigen and Manning, 1975; Bonaventura et al., 1976; Adachi et al., 1983). 3) His 146 $\beta$  (Rollema et al., 1975; Adachi et al., 1983; Matsukawa et al., 1984). Chloride binding to the high-affinity sites (Val 1 $\alpha$ , His 146 $\beta$ ;  $K_D=0.01$  M) and to other, weaker sites ( $K_D=10$  M; possibly Lys 82 $\beta$ ; see Perutz et al., 1980) has been measured by  $^{35}\text{Cl}$ -NMR (Chiancone et al., 1972, 1975). Chiancone et al. (1975) suggest that the involvement of  $\text{Cl}^-$  in the binding of  $\text{H}^+$  is fairly direct. For example, they propose that lowering the pH to 6.0 enhances  $\text{Cl}^-$  binding to deoxy Hb because the carboxyl group of Asp 94 $\beta$ , which is normally salt-bridged to His 146 $\beta$ , is partially neutralized, enabling  $\text{Cl}^-$  to bind to a site on His 146 $\beta$ . Chloride binding to a basic group also would be expected to raise the  $pK_a$  of that group, thereby stabilizing  $\text{H}^+$  binding (Rollema et al., 1975; O'Donnell et al., 1979).

In hemoglobins of certain mammals other than man, the effect of  $\text{Cl}^-$  on the pH-dependent  $\text{O}_2$  affinity can be very pronounced (Bonaventura et al., 1974; Fronticelli et al., 1984). In lemur Hb, for example,  $\text{Cl}^-$  significantly enhances the  $\text{O}_2$  affinity at  $\text{pH}>6$  to nearly the same extent as the effector 2,3-Diphosphoglycerate (Bonaventura et al., 1974).

The degree of similarity between the  $\text{Cl}^-$  binding properties of Hb and those of the OEC is not entirely discernible, since it is not yet known whether  $\text{Cl}^-$  controls the rate of  $\text{O}_2$ -evolution by an allosteric mechanism, or whether amino acid salt-bridges are involved at all in  $\text{Cl}^-$  binding. Because of the strong linkage between  $\text{Cl}^-$ ,  $\text{H}^+$ , and conformation, it nevertheless has the potential for being a useful

system for comparison.

Halorhodopsin (HR) and the erythrocyte Band 3 anion transport protein (Band 3 protein) are membrane-bound translocators of  $\text{Cl}^-$  and other anions. HR is a light-driven pump which moves  $\text{Cl}^-$  across the bacterial membrane (Schobert and Lanyi, 1982).  $^{35}\text{Cl}$ -NMR binding studies revealed that the protein has two  $\text{Cl}^-$  binding sites: a high affinity site with  $K_D=135-150$  mM and a low-affinity site with  $K_D \gg 1$  M (Falke et al., 1984a). One of the sites (Site I) is relatively non-specific for  $\text{Cl}^-$  and will bind many other monovalent anions (Schobert and Lanyi, 1986). This site is near, but not on, the retinal Schiff base chromophore (Steiner et al., 1984; Maeda, et al., 1985; Schobert and Lanyi, 1986). Chloride binding to this site raises the  $\text{p}K_a$  of the Schiff base by about 2 units (Steiner et al., 1984; Schobert and Lanyi, 1986; Schobert et al., 1986) and stabilizes the 580 nm intermediate in the photocycle (Steiner et al., 1984; Lanyi and Vodyanoy, 1986). Both the binding constant of the anion and the observed  $\Delta\text{p}K_a$  of the Schiff base decrease sharply as the Stokes radius of the anion increases (Schobert and Lanyi, 1986). These effects are best explained by an electrostatic interaction between the anion and the protein which depends on the size of the anion and which raises the  $\text{p}K_a$  of the Schiff base (Schobert and Lanyi, 1986).

The other anion binding site (Site II) has a greater specificity for  $\text{Cl}^-$  (Steiner et al., 1984; Schobert and Lanyi, 1986). Displacement of  $\text{Cl}^-$  from Site I by other anions may block access to this second site (Steiner et al., 1984).

Chloride binding to the Band 3 protein (for a review, see Passow et al., 1980) has been studied by a variety of methods, including amino acid modification and  $^{35}\text{Cl}$ -NMR. This protein translocates  $\text{Cl}^-$  across

the red cell membrane by a sequence of conformational changes. An important group controlling the activity of the protein is a specific lysine residue known as Lys a, which is located near the outward face of the protein and whose  $\epsilon$ -amino group has a lower  $pK_a$  (8.5) than those on most other lys groups in the protein. This Lys and another nearby Lys (Lys b,  $pK_a=8.8$ ) form part of a cluster of five basic amino acid residues. Due to their close proximity, their  $pK_a$ 's are shifted downward from the  $pK_a$  of 10.6 that would be expected for  $\epsilon$ -amino groups (Passow et al., 1980). Although this cluster may be involved in the electrostatic accumulation of  $Cl^-$ , its direct involvement as part of the substrate binding/translocating site is not clear (Knauf and Grinstein, 1982; Passow et al., 1982). The pH behavior of the substrate site is more consistent with the presence of a guanidinium group of arginine (for a discussion, see Wieth et al., 1980; Knauf and Grinstein, 1982). Another positively charged region near the protein surface has been suggested to function as a modifier site which regulates the overall activity (Passow et al., 1982; Knauf and Grinstein, 1982; see also Macara and Cantley, 1981).

Falke and Chan (1985) examined  $Cl^-$  binding to the inward- and outward-facing transport sites by  $^{35}Cl$ -NMR and determined a  $K_D$  for  $Cl^-$  of about 90 mM. Other monovalent anions, such as  $I^-$ ,  $HCO_3^-$ , and  $F^-$  compete with  $Cl^-$  at these sites with relative affinities in the order  $I^- > HCO_3^- > Cl^- > F^-$  (Falke et al., 1984b). These and other  $^{35}Cl$ -NMR studies failed to identify a modifier site, and therefore a ping-pong mechanism with a single  $Cl^-$  binding site has recently been proposed (Falke et al., 1984b,c; 1985; Falke and Chan, 1985).

## I. Conclusions

Comparison of what is currently known about  $\text{Cl}^-$  binding to various proteins with what is known about  $\text{Cl}^-$  behavior in the OEC provides several insights into the potential structure and operation of the photosynthetic system. It seems clear from this analysis that the most likely location for reversible, pH-dependent, high-affinity  $\text{Cl}^-$  binding is a basic amino acid residue or residues, and not a metal center. Only the direct association of  $\text{Cl}^-$  with a protein is capable of producing the alkaline shift of the activity-pH optimum that was first observed by Gorham and Clendenning and described in general theoretical terms by Massey (see section F). This effect is observed in nearly all of the proteins reviewed in section H. Every one of these proteins is believed to bind  $\text{Cl}^-$  at a basic amino acid residue.

Upon more detailed examination, however, it is clear that the pH-dependent behavior of  $\text{Cl}^-$  in spinach thylakoids and PS II membranes does not seem to resemble any one of the binding sites from these other proteins. In this respect, Homann's paradoxical finding that  $\text{Cl}^-$  activation has a  $\text{pK}_a=6.0$ , but that  $\text{Cl}^-$  release has a  $\text{pK}_a>8$  (section F) may be an important clue. Resolution of this problem may be possible if one assumes that the OEC  $\text{Cl}^-$  binding/activation mechanism actually involves a hybrid structure. Thus, the active-site effect of  $\text{Cl}^-$  in the OEC may involve binding at or near a group of histidyl residues which, as in RNase A, have  $\text{pK}_a$ 's=6 and are involved in  $\text{H}^+$  abstraction (Coleman and Govindjee, 1985; Homann, 1987) and donation/transfer (giving rise to a  $\text{Cl}^-$  sensitive, bell-shaped pH optimum). In the case of the OEC, however, the  $\text{H}^+$  would be abstracted from water and then possibly donated to another protein. Since  $\text{Cl}^-$  binding is capable of stabilizing a positive charge ( $\text{H}^+$ ), it could assist in stabilizing transiently-

protonated bases, thereby allowing the S-states to advance as  $H^+$  is released. (For earlier discussions, see Govindjee et al., 1983, and Crofts and Wraight, 1983.)

A second set of sites, with greater resemblance to those in angiotensin-converting enzyme,  $\alpha$ -amylase, serum albumin, and hemoglobin, appears to have  $Cl^-$  binding residues with high  $pK_a$ 's (lysyl and arginyl). As in these isolated proteins,  $Cl^-$  binding to such sites in the OEC involves the sequestering of  $H^+$ , indicating that inter- and/or intra-subunit salt-bridges may be involved. The presence of a number of these secondary sites would explain why actual removal of most of the  $Cl^-$  from the OEC requires high pH treatment. A  $pK_a$  for these groups of  $\sim 8$  would be consistent with their incorporation into positively-charged clusters (as in Band 3) and/or with the proximity of bound  $Ca^{2+}$  (as in  $\alpha$ -amylase).

A role for anions such as  $Cl^-$  in raising the  $pK_a$ 's of protonatable groups at the active site may explain the observation that activity depends on the size of the anion, since, as was discovered in halorhodopsin, the magnitude of the electrostatic interaction between the anion and the bound positive charge may determine the degree to which the  $pK_a$ 's in the vicinity are raised (see also Matthew and Richards, 1982, for RNase A).

Finally, it should be noted that the stabilization against denaturation which is contributed to the OEC by  $Cl^-$  binding is typical of the effects of  $Cl^-$  binding to  $\alpha$ -amylase and other  $Cl^-$  activated enzymes (see also Suzuki and Suzuki, 1974).

## J. Objectives of This Thesis

The experiments reviewed in sections A-G of this chapter show that much is known about the kinetics of  $\text{Cl}^-$  activation of the OEC and about the polypeptides involved in  $\text{Cl}^-$  binding. However, the review of other  $\text{Cl}^-$  binding proteins in section H demonstrates that without direct measurements of  $\text{Cl}^-$  binding it is difficult to specify the location of the binding sites and to elucidate the overall mechanism. The objective of the experiments described in this thesis is to combine direct measurement of  $\text{Cl}^-$  binding (by  $^{35}\text{Cl}$ -NMR) with assays of steady-state  $\text{O}_2$ -evolution in order to determine: 1) the number of  $\text{Cl}^-$  binding sites and their effect on  $\text{O}_2$ -evolution in the native complex; 2) the role of  $\text{Cl}^-$  in maintaining the stability of the OEC; 3) the precise location of the  $\text{Cl}^-$  binding sites with respect to the OEC polypeptides and the catalytic Mn; and 4) the mechanism by which  $\text{Cl}^-$  stimulates  $\text{O}_2$ -evolution. These investigations will be described in the remaining chapters.

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## II. $^{35}\text{Cl}$ -NMR MEASUREMENT OF CHLORIDE BINDING TO THE OXYGEN-EVOLVING COMPLEX OF SPINACH PHOTOSYSTEM II

### A. Introduction

The oxygen-evolving complex (OEC) of Photosystem II (PS II) in green plants requires chloride in order to catalyze the light-driven oxidation of water with high efficiency (Kelley and Izawa, 1978; Hind et al., 1969; Heath and Hind, 1969; Izawa et al., 1969; Critchley et al., 1982). The requirement for  $\text{Cl}^-$  is not exclusive, since other monoanions also activate the enzyme. Their effectiveness follows the order  $\text{Cl}^- > \text{Br}^- \gg \text{NO}_3^- > \text{I}^-$  (Hind et al., 1969; Kelley and Izawa, 1978; Critchley et al., 1982). Although many details are known about the kinetics of  $\text{Cl}^-$  activation (for reviews, see Critchley, 1985; Govindjee et al., 1985; Homann, 1987; and Chapter 1), the information gained through this kind of study is not, by itself, sufficient to construct a model for the mechanism of  $\text{Cl}^-$  activation. In a system so structurally intricate as the OEC, it is important to know both the number of  $\text{Cl}^-$  binding sites and their location within PS II.

$^{35}\text{Cl}$ -NMR has been widely used to examine the binding of  $\text{Cl}^-$  to various proteins (Chiancone et al., 1972, 1975; Norne et al., 1975; Falke et al., 1984). Chloride is not a very sensitive NMR nucleus, however. For this reason, the first NMR observations of  $\text{Cl}^-$  binding to thylakoid membranes were performed on preparations from the leaves of extremely salt-tolerant plants (halophytes), since they have a high  $\text{Cl}^-$  requirement for  $\text{O}_2$ -evolution (Critchley et al., 1982; Bălanu et al., 1984). In the case of thylakoids from spinach, the apparent activator dissociation constant for  $\text{Cl}^-$  ( $K_A'$ ) is only 0.6-0.9 mM (Kelley and Izawa, 1978; Critchley et al., 1982), and thus  $^{35}\text{Cl}$ -NMR binding studies

on them have only recently become feasible because of advances in instrumentation. We therefore present here the first direct measurements of  $\text{Cl}^-$  binding to  $\text{Cl}^-$  depleted spinach thylakoids and PS II membranes in the  $\text{Cl}^-$  concentration range where  $\text{O}_2$ -evolution is most sensitive to the activator.

## B. Materials and Methods

Thylakoid Preparation. Thylakoids and PS II membranes were isolated from market spinach. Care was taken to avoid unnecessary illumination during the preparation, particularly with  $\text{Cl}^-$  depleted samples. Thylakoids were prepared by mixing washed spinach leaves (chopped and de-veined) in ice-cold grinding buffer consisting of 2 mM ethylene diamine tetraacetic acid (EDTA), 0.1% bovine serum albumin (BSA), and 50 mM sodium phosphate or hydroxyethyl piperazine ethane sulfonic acid (HEPES) (pH 7.2) at a proportion of 2 ml buffer per g leaves. After grinding for 8 s at high speed in a Waring blender, the homogenate was filtered through 12 layers of cheesecloth into an equal volume of chilled buffer without BSA. For large batches, the grinding and filtering was done in stages.

Chloride Depletion of Thylakoids. After the filtrate was centrifuged briefly at low speed to remove any remaining debris, it was centrifuged at 6,000 x g for 12 minutes to pellet the thylakoids. For thorough  $\text{Cl}^-$  depletion, the thylakoids were resuspended and diluted to about  $50 \mu\text{g Chl ml}^{-1}$  in a large volume of buffer containing  $1.0 \mu\text{M}$  Gramicidin D (freshly added) and 20 mM bis-tris propane/ morpholino-propane sulfonic acid (BTP/MOPS), pH 8.6 (see Theg et al., 1982). This suspension was allowed to stir on ice in darkness for 30 min. The

thylakoids were then pelleted by centrifugation as before and resuspended to 3.5 mg Chl ml<sup>-1</sup> in 50 mM HEPES, pH 7.2. Thylakoid samples for <sup>35</sup>Cl-NMR or activity measurements were placed on ice and used immediately after preparation.

Photosystem II Preparation. PS II membranes were prepared by a modification of the method of Berthold et al. (1981). Thylakoids were prepared as described above; however, in order to avoid any inactivation prior to the incubation in Triton X-100, 50 mM NaCl was added to both isolation buffers, along with 100mM sucrose in the grinding buffer. Thylakoids taken from the first high-speed centrifugation step were resuspended to 2.0 mg Chl ml<sup>-1</sup> in buffer containing 5 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 2 mM sodium ascorbate (freshly added), and 20 mM morpholinoethane sulfonic acid (MES), pH 6.0. Triton X-100 (Calbiochem-Behring) was added to a final concentration of 25 mg / mg Chl. The suspension was stirred on ice in darkness for 25 min, transferred to centrifuge tubes, and centrifuged for 25 min at 40,000 x g.

Chloride Depletion of PS II Membranes. The uppermost pellet was rinsed and then resuspended in 400 mM sucrose, 20 mM MES (pH 6.0), and then centrifuged again for 20 min. This washing-centrifugation cycle was repeated twice more to remove any loosely-bound Cl<sup>-</sup>. Treatment with sodium sulfate was found to be effective at removing residual Cl<sup>-</sup> from the membranes (as determined by assaying activity in the absence of Cl<sup>-</sup>), but also tended to lower slightly the maximum activity attainable in 50 mM Cl<sup>-</sup>. For this reason, a brief pH-jump treatment was used instead (Homann, 1985). In this procedure, the washed PS II membranes were resuspended in a small volume of buffer at pH 6.0 and then diluted to about 200 μg chlorophyll (Chl) ml<sup>-1</sup> in 20 mM BTP/MOPS for 20 s at a final pH of 8.2. This suspension was then quickly returned to pH 6.0 by

dilution with a measured amount of 50 mM MES, 400mM sucrose at pH 5.5. The  $\text{Cl}^-$  depleted PS II membranes were centrifuged a final time and resuspended to  $2.5 \text{ mg Chl ml}^{-1}$  in the  $\text{Cl}^-$  free buffer (pH 6.0). Finally, the sample was distributed into measured aliquots and frozen immediately in liquid  $\text{N}_2$ , where it was stored until use. Chl concentration was measured by the method of Graan and Ort (1984).

Hill Reaction Measurements. The Hill activity of the thylakoids was measured by 2,6-Dichlorophenolindophenol (DCIP) reduction. Thylakoids were suspended at  $5.8 \mu\text{g Chl ml}^{-1}$  in buffer containing  $1.0 \mu\text{M}$  Gramicidin D,  $50 \mu\text{M}$  DCIP, and 50 mM HEPES (pH 7.2), along with a measured amount of NaCl. In order to minimize random errors in  $[\text{Cl}^-]$ , a set of assay buffers for the activation curves was prepared by proportional dilution of two assay buffer stocks, one without  $\text{Cl}^-$  and one with the maximal concentration of  $\text{Cl}^-$ . Thylakoids were allowed to incubate in the assay buffer in the cuvette for 3 min before illumination. The apparent level of  $\text{Cl}^-$  depletion (measured by comparing the Hill activity  $\pm 50 \text{ mM Cl}^-$ ) was approximately 67%.

DCIP reduction (at pH 7.2) was measured in a stirred cuvette at 595 nm in a Cary-14 spectrophotometer equipped with an exciting light (see Armstrong, 1964, for a discussion of the pH-dependence of DCIP). The exciting light, which was generated by a small tungsten-halogen lamp (Sylvania DVY), was passed through a 4-inch water filter, a glass heat filter, and a Corning C.S. 2-58 red glass filter (50% transmission at 645 nm). It was focused onto the sample holder by a series of lenses. The light intensity at the sample holder was measured with a YSI-Kettering Model 65 Radiometer. Light intensity was varied by using a series of metal-coated neutral density filters (Melles-Griot). The

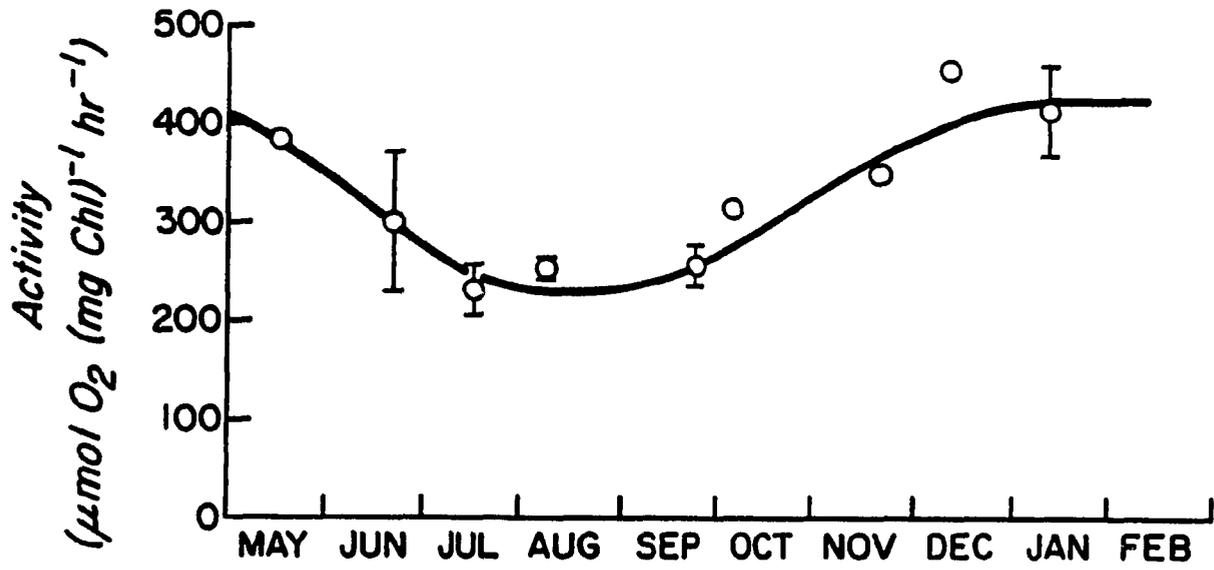
optical density of each filter was measured at the appropriate wavelengths.

The activity of the PS II membranes was measured in a Hansatech oxygen electrode maintained at 25°C by a bath-circulator. The exciting light was provided by a Kodak carousel 4200 slide projector, and was passed through a Corning C.S. 3-69 yellow filter (50% transmission at 530 nm) and 2 inches of aqueous 1% CuSO<sub>4</sub>. The assay suspension contained 20 μg Chl ml<sup>-1</sup> in buffer containing 400 mM sucrose, 3.0 mM potassium ferricyanide, 0.4 mM 2,6-Dichlorobenzoquinone (DCBQ), and 20 mM MES (pH 6.0). NaCl was added where appropriate. The suspension was allowed to stir in darkness for 3 min before illumination. The level of apparent Cl<sup>-</sup> depletion for PS II membranes was approximately 20–25% when assayed 3 min after dilution into the cuvette. This level increased to about 45% when the mixture was allowed to stir for 20 min before illumination. Although this result indicates that the PS II membranes retain a small amount of tightly-bound Cl<sup>-</sup> at the active site, this remaining pool eventually equilibrates with the buffer. The timescale of the NMR measurement is long enough for this phenomenon not to affect the binding curve.

A surprising finding was that the maximum activity for the PS II membranes in 50 mM Cl<sup>-</sup> was found to vary over the course of a year (Fig. 2), with the lowest activity being observed during the summer (June to October). The reason for this variation is unknown. It may reflect genetic or developmental differences, or seasonal differences in growing and shipping conditions.

Sample Preparation for NMR Measurements. For <sup>35</sup>Cl-NMR experiments, 1.6 ml aliquots of frozen PS II membranes were partially thawed by passing each cryotube repeatedly under hot running water until the

Figure 2. Oxygen-evolution from spinach PS II membranes at different times of the year.  $\text{Cl}^-$  depleted PS II preparations were assayed in 50 mM NaCl, as described in Materials and Methods. Points with error bars represent the mean activities for 2-4 different preparations.



contents were loosened from the walls. The contents were then rapidly thawed and diluted by mixing them into 6.4 ml of buffer containing 400 mM sucrose and 20 mM MES (pH 6.0). Measurements were made at pH 6.0 because the results of Homann (1985) indicated that  $\text{Cl}^-$  binds to groups on the membrane with a  $\text{pK}_a$  of about 6. NaCl was added by syringe from appropriate concentrated stock solutions (prepared by serial dilution). Thylakoids were simply diluted into a larger volume of buffer before NaCl addition. All manipulations were performed in darkness, and a new sample was used for each measurement.

Bovine serum albumin (an ultra-pure grade) for NMR measurements was purchased from Boehringer Mannheim. Sodium bromide (spectroscopic grade, >99.999%) was purchased from Aldrich.

NMR Measurements.  $^{35}\text{Cl}$ -NMR measurements were carried out at 19°C on a home-built 250 MHz spectrometer using a specially-designed sideways-spinning probe (see Oldfield and Meadows, 1978) capable of holding a 20mm (7.8 ml) sample cell. Samples were spun at relatively low speed to maintain the homogeneity of the suspension, but care was taken not to centrifuge the sample onto the walls of the cell, particularly in the case of thylakoids. Spectra were obtained at 24,508 MHz using a 33  $\mu\text{s}$  90° pulse and 360 ms recycle time. Signals were detected in quadrature with 32K data points and a spectral width of  $\pm 25,000$  Hz. They were then block averaged (500 scans per block) and transferred to a hard disk by a Nicolet 1180E computer. The data points were multiplied by an exponential line-broadening factor to reduce the noise. This added 15 Hz to the observed linewidth for samples in 0.1 mM to 0.5 mM  $\text{Cl}^-$  and 10 Hz to those in 0.6 to 20 mM  $\text{Cl}^-$ . The data were then Fourier-transformed into the frequency domain. The observed linewidth for each sample was measured at half-maximum intensity with a ruler and compared against a

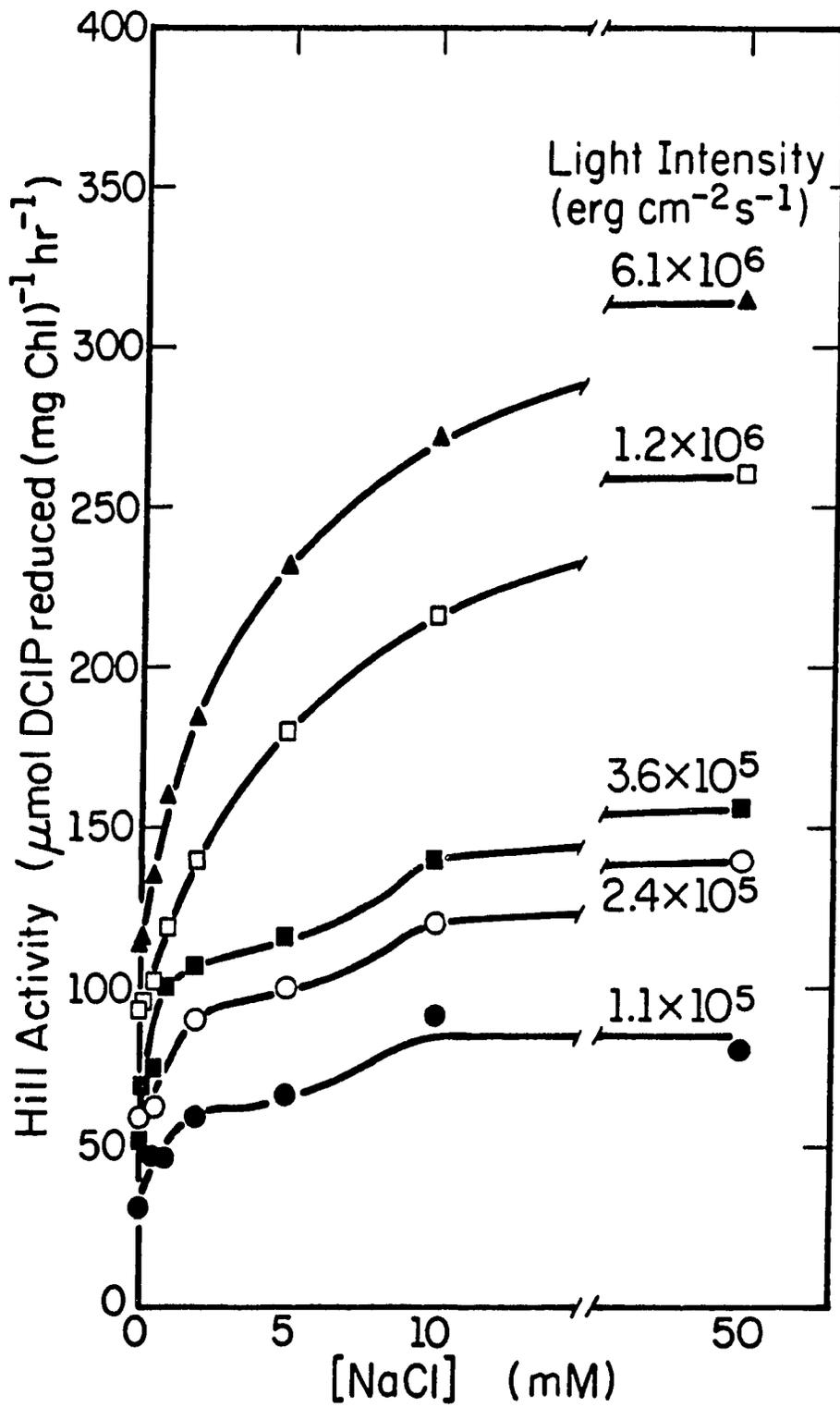
computer-fit of the lineshape. The linewidth for free  $\text{Cl}^-$  in solution was measured by taking the spectra of buffer solutions containing 10 mM  $\text{Cl}^-$ . There was no detectable concentration dependence of the linewidth for these solutions in the range of 0.1 to 10 mM  $\text{Cl}^-$ . Spectra of these standard solutions were recorded periodically in order to check the field homogeneity during the runs. The computer-calculated S/N for a 10 mM  $\text{Cl}^-$  buffer standard after 1,000 accumulations with no line-broadening was 58:1. The net or excess linewidth ( $\Delta\nu_t$ ) was calculated by subtracting the linewidth for the  $\text{Cl}^-$  buffer standard from the observed linewidth for the membrane suspension (Balanu et al., 1984). Values of  $\Delta\nu_t$  obtained at each  $[\text{Cl}^-]$  for different membrane preparations were averaged without normalization. Error bars represent the sample standard error of the mean.

### C. Results

In order to determine the effect of  $\text{Cl}^-$  depletion on the efficiency of steady-state electron transport from water to DCIP, we measured the Hill activity in thylakoids as a function of  $[\text{Cl}^-]$  at various light intensities (Fig. 3). At high light intensity, the activity increases in a smooth hyperbola as the  $[\text{Cl}^-]$  is increased from 0 (no NaCl addition) to 50 mM. This is consistent with earlier measurements of the effect of  $\text{Cl}^-$  on  $\text{O}_2$ -evolution (Kelley and Izawa, 1978). However, at low light intensity (5.5% of saturating and below) the activation curves begin to show a non-hyperbolic dependence on  $[\text{Cl}^-]$ . The deviations from hyperbolic behavior are most prominent at  $[\text{Cl}^-] < 10$  mM.

We examined this phenomenon more closely by focusing on one of the curves (5.5% saturating light intensity) in the region from 0 to 10-20

Figure 3.  $\text{Cl}^-$  activation of the Hill reaction in thylakoids at various light intensities. The intensities listed with each curve represent (from top to bottom) 93%, 18%, 5%, 4%, and 2% of saturating light intensity, respectively.



mM  $\text{Cl}^-$  (Fig. 4). In order to see the points more easily at low  $[\text{Cl}^-]$ , these results are also plotted as a function of  $[\text{Cl}^-]$  on a log scale in Fig. 5. In this experiment, we were able to observe at least three intermediary plateaus in the curve (centered at approximately 0.5 mM, 1.5 mM, and 4.5 mM  $\text{Cl}^-$ ) along with increases in slope (at approximately 0.75-0.9 mM, 2.5 mM, and 5.0 mM  $\text{Cl}^-$ ) and decreases in slope (at approximately 0.25-0.3 mM, 1.0 mM, 3.5 mM, and 6.0-7.0 mM  $\text{Cl}^-$ ). A plot of this data in the form of activity vs.  $\log [\text{Cl}^-]$  (Fig. 5), which for a typical Michaelis-Menten system gives a smooth sigmoid (Metzler, 1977), shows clear deviations from simple behavior. It is therefore impossible to define a single activator dissociation constant ( $K_A'$ ) for  $\text{Cl}^-$  in this system. The first three "steps" in the curve contribute 47%, 27%, and 8%, respectively, to the amount of activity that is stimulated by  $\text{Cl}^-$  addition. The apparent affinity for  $\text{Cl}^-$  appears to decrease as the  $[\text{Cl}^-]$  increases, suggesting that negative cooperativity might be involved in the overall mechanism.

In thylakoids in which the OEC has been inactivated by heating,  $\text{Cl}^-$  has no effect on the diphenyl carbazide (DPC)  $\rightarrow$  DCIP reaction, indicating that at this light intensity,  $\text{Cl}^-$  does not effect the rate limitation on electron transport beyond the OEC (Fig. 6). DPC is known to donate electrons at Z, the electron donor to  $\text{P680}^+$ , bypassing the OEC (Izawa, 1980).

Since we had observed that the appearance of the plateaus in the  $\text{Cl}^-$  activation curve depends on the light intensity, we decided to examine the effect of brief pre-illumination (5 s at 30% of saturating light intensity) on this system. Figures 7 and 8 show that a brief pre-illumination significantly reduces the deviations from hyperbolic behavior, particularly at low  $[\text{Cl}^-]$ . At higher  $[\text{Cl}^-]$ , the relative

Figure 4.  $\text{Cl}^-$  activation of the Hill reaction in thylakoids at 5.5% of saturating light intensity ( $3.8 \times 10^5 \text{ erg cm}^{-2}\text{s}^{-1}$ ). Arrows indicate the locations of the intermediary plateaus. The maximum activity (at 20 mM  $\text{Cl}^-$ ) was  $252 \mu\text{mol DCIP reduced (mg Chl)}^{-1} \text{ hr}^{-1}$ . Error bars show the sample standard error for separate measurements on two different preparations.

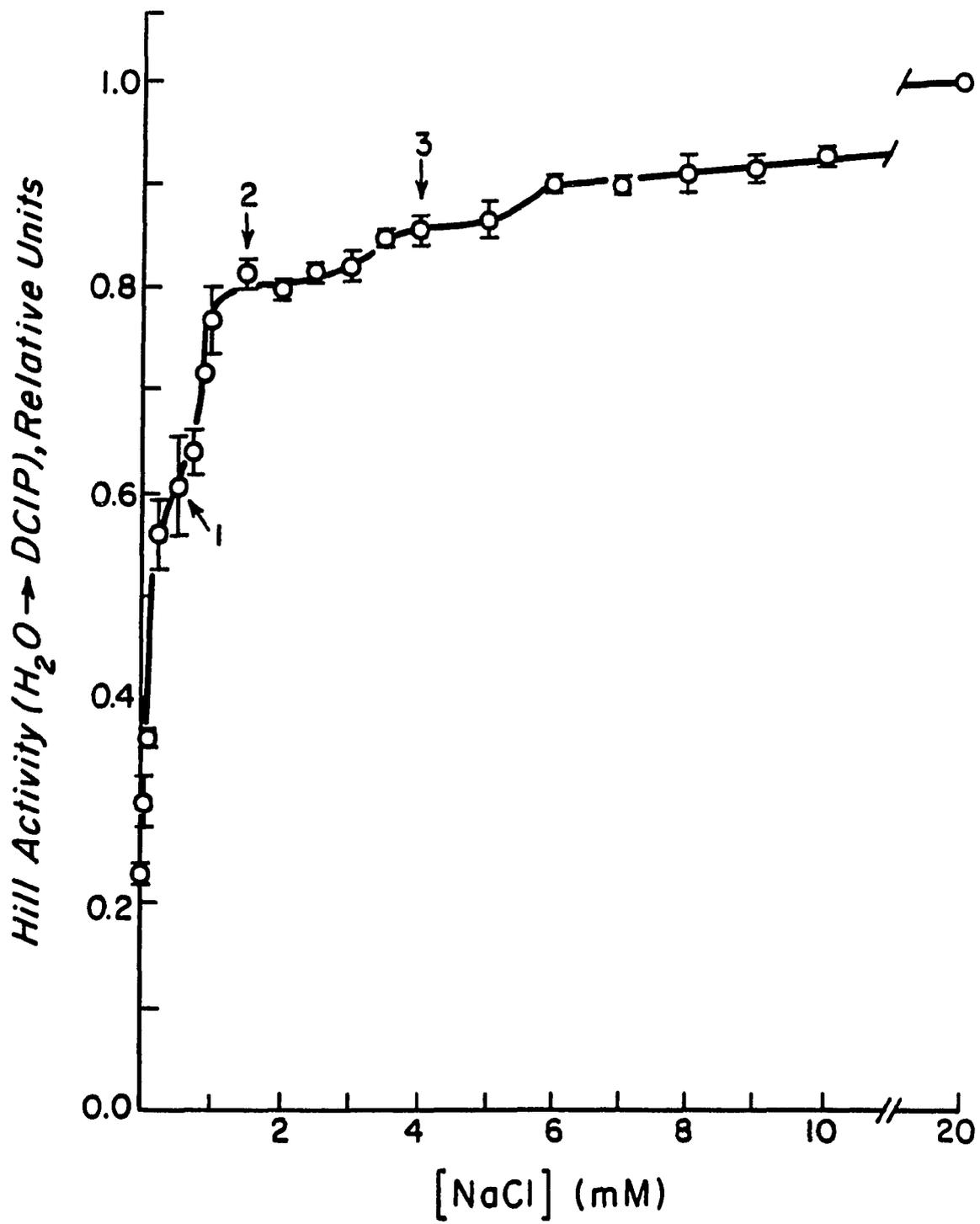


Figure 5. Relative Hill activity vs.  $\log [Cl^-]$  for spinach thylakoids at 5.5% of saturating light intensity. Chloride concentrations marked with arrows (top) refer to corresponding points shown later in Fig. 12. See Fig. 4 for other details.

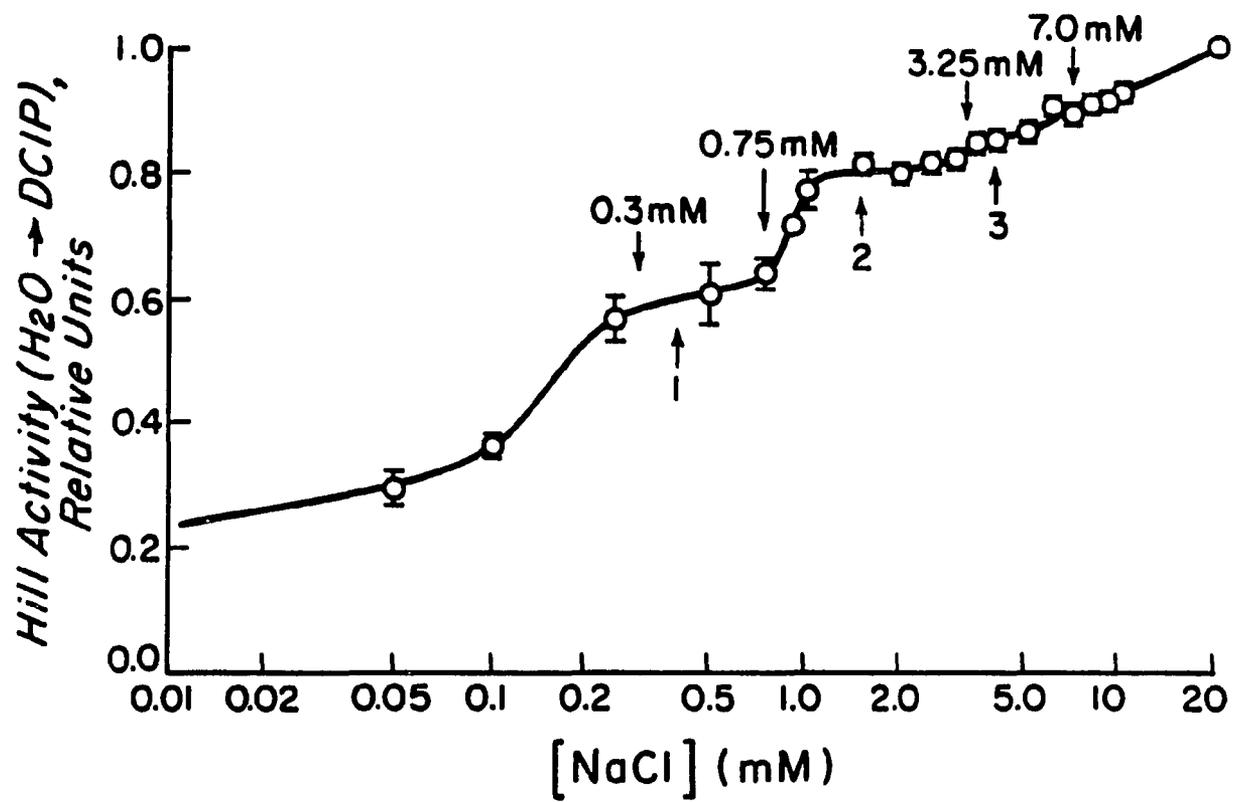


Figure 6. Effect of  $\text{Cl}^-$  on the rate of electron flow from DPC to DCIP for thylakoids heated at  $45^\circ\text{C}$  for 5 min. This reaction bypasses the OEC. The DPC concentration was 1.0 mM. The assay pH was 7.2.

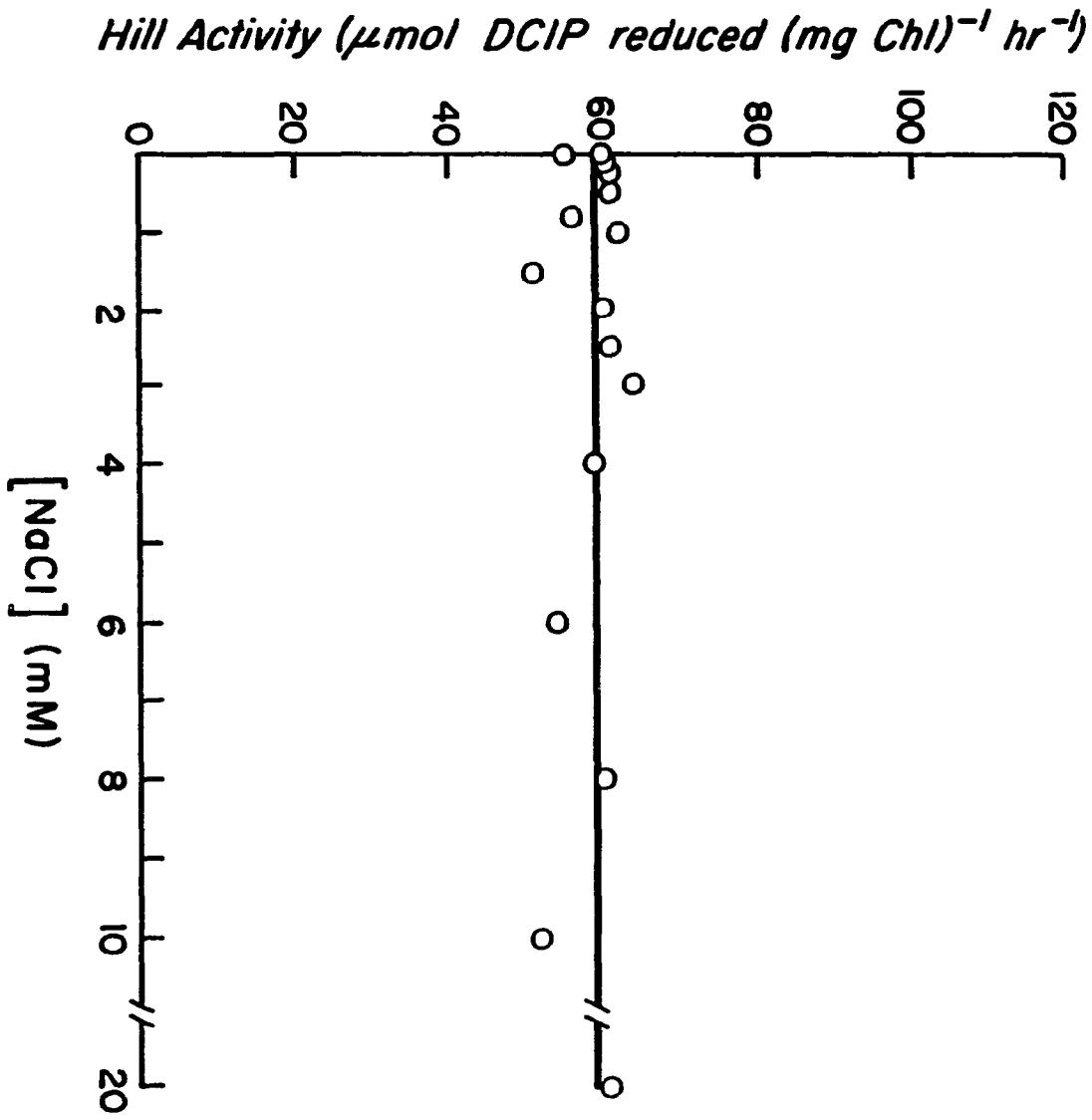


Figure 7.  $\text{Cl}^-$  activation of the Hill reaction in thylakoids pre-illuminated prior to  $\text{Cl}^-$  addition.  $\text{Cl}^-$  depleted thylakoids were suspended in assay buffer (minus  $\text{Cl}^-$ ) in a cuvette and illuminated with the exciting light (at  $2.0 \times 10^6 \text{ erg cm}^{-2} \text{ s}^{-1}$ , 30% of saturating) for 5 s.  $\text{Cl}^-$  was immediately added to the cuvette in darkness. The activity was assayed 3 min after the pre-illumination. The maximum activity at 20 mM  $\text{Cl}^-$  was  $249 \mu\text{mol DCIP reduced (mg Chl)}^{-1} \text{ hr}^{-1}$ . Light intensity for the assay was the same as in Fig. 4. Arrows indicate possible plateaus.

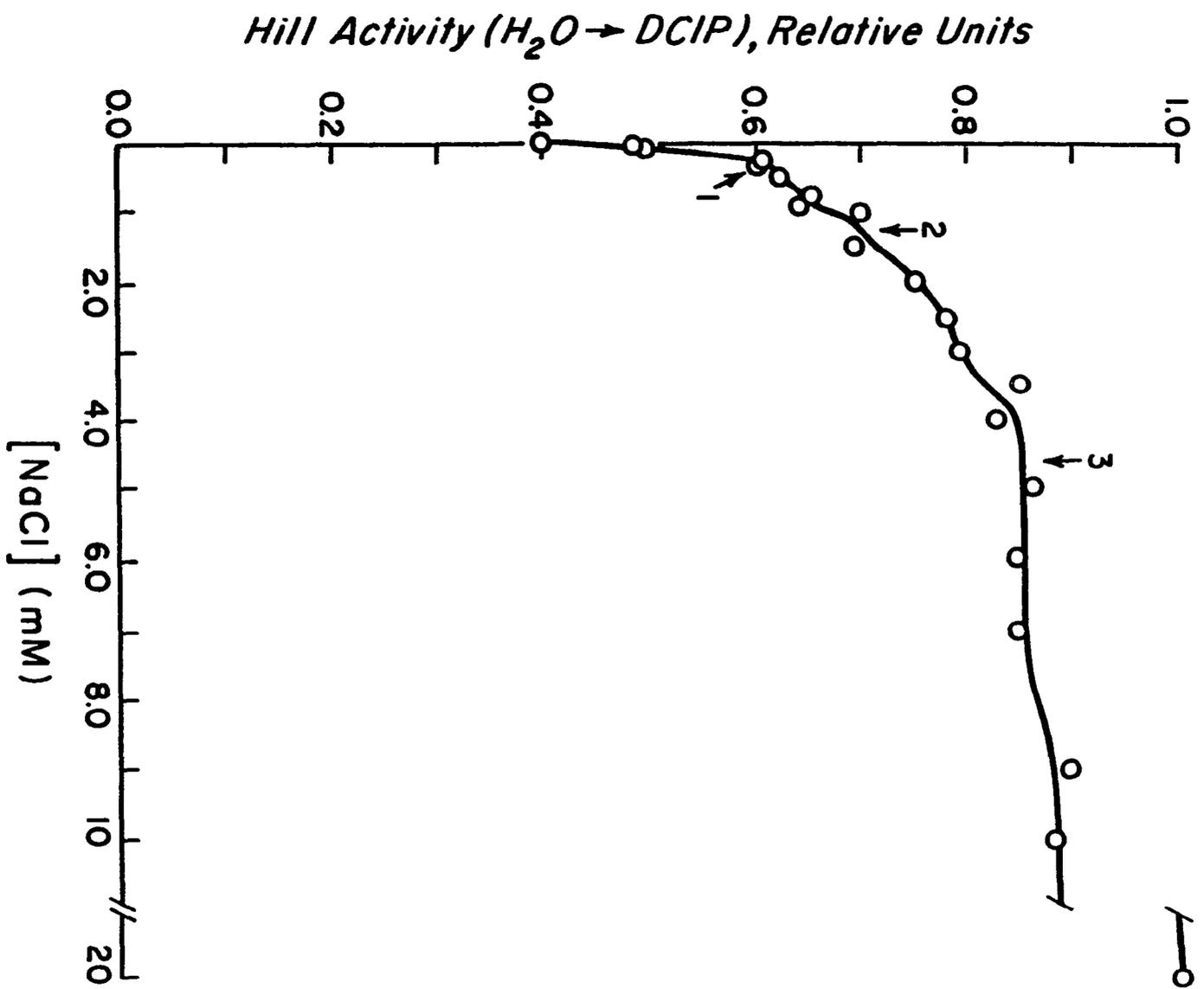
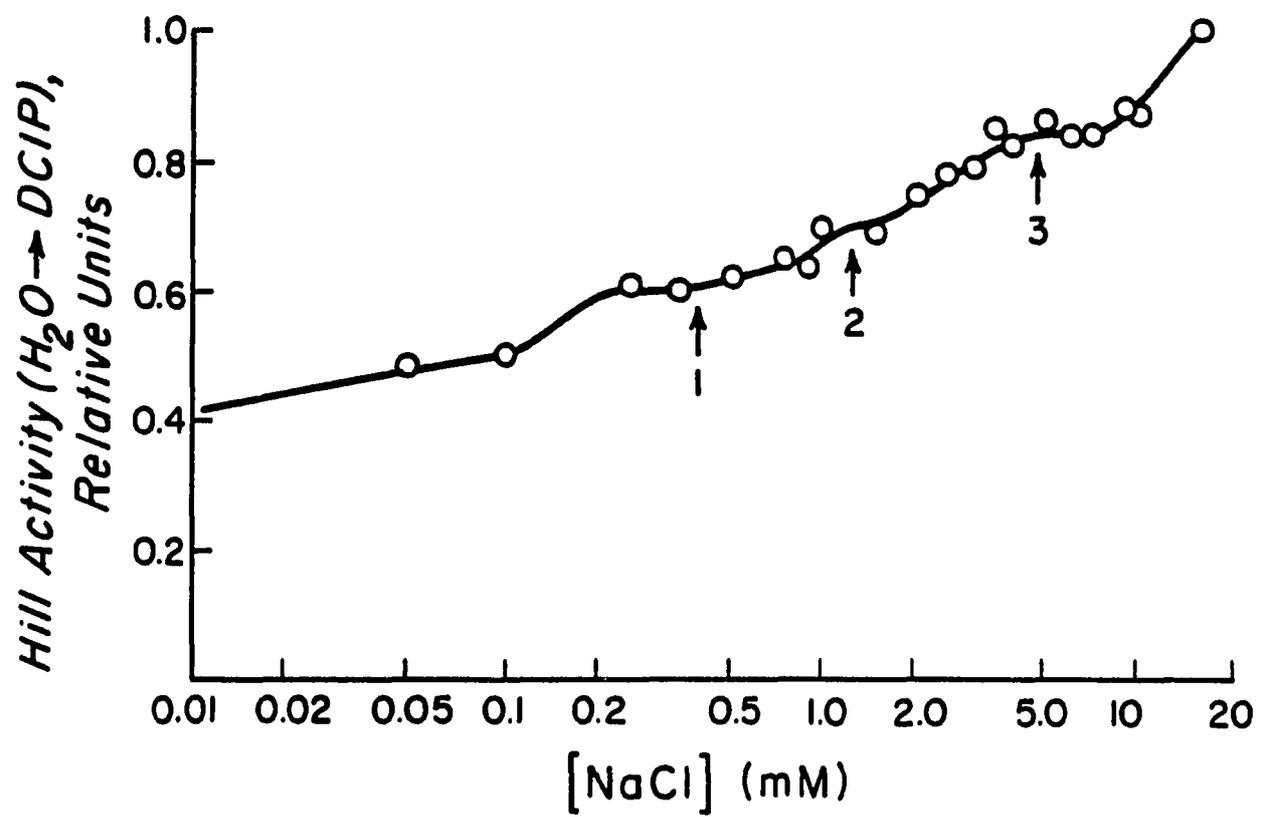


Figure 8. Relative Hill activity vs.  $\log [Cl^-]$  for pre-illuminated thylakoids. See Fig. 7 for other details.



activity is slightly reduced, probably as a result of a diminution in the proton gradient across the thylakoid membrane. The persistent effect of illumination on the apparent dissociation constant for  $\text{Cl}^-$  indicates that this treatment influences the apparent binding of the anion, either by a conformational change in the OEC or by protonation of  $\text{Cl}^-$  binding sites.

The appearance of intermediary plateaus (i.e., complex kinetic cooperativity) in the  $\text{Cl}^-$  activation curve for the Hill reaction indicated not only that multiple  $\text{Cl}^-$  binding sites might be involved, but also that  $\text{Cl}^-$  may bind cooperatively to these sites (Segel, 1975; Kurganov, 1982). In order to investigate the  $\text{Cl}^-$  binding properties of the OEC by an independent and more direct method, we measured the  $\text{Cl}^-$  concentration dependence of the  $^{35}\text{Cl}$ -NMR linewidth in both thylakoids and PS II membranes. For a system in which a  $\text{Cl}^-$  ion bound to a protein or other macromolecule exchanges rapidly with  $\text{Cl}^-$  in solution, the following equation can be written (Baiaru et al., 1984):

$$\Delta\nu_{\text{obs}} = \Delta\nu_b f_b + \Delta\nu_f (1-f_b), \quad (1)$$

where  $\Delta\nu_{\text{obs}}$ , the observed  $^{35}\text{Cl}$ -NMR linewidth at half-maximum intensity, is the weighted average of the contributions from  $\text{Cl}^-$  in the bound state ( $\Delta\nu_b$ ) and the free state ( $\Delta\nu_f$ ). The contribution from each site depends on the fraction of  $\text{Cl}^-$  bound ( $f_b$ ). It is more useful, however, to plot the net or excess linewidth ( $\Delta\nu_t$ ), which reflects the amount of  $\text{Cl}^-$  actually bound. Thus, since  $\Delta\nu_b$  (approximately 10 kHz) is very much larger than  $\Delta\nu_f$  (approximately 12-30 Hz, depending on the viscosity), and  $f_b \ll 1$  (for a dilute protein solution), we can write:

$$\Delta\nu_t = \Delta\nu_{\text{obs}} - \Delta\nu_f \approx \Delta\nu_b f_b. \quad (2)$$

For a simple system, eqn. 2 would be expected to produce a binding curve for  $\Delta\nu_t$  vs.  $[\text{Cl}^-]$  in the form of a smoothly descending hyperbola, since  $f_b$  decreases with increasing  $[\text{Cl}^-]$ . This type of curve has been observed for many  $\text{Cl}^-$ -binding enzymes, including those in which more than one  $\text{Cl}^-$  binding site is present (see, e.g. Baianu et al., 1984; Coleman and Govindjee, 1987; and NMR refs. in Chapter 1, sections F and H).

In  $\text{Cl}^-$  depleted thylakoids and PS II membranes, however, the  $^{35}\text{Cl}$ -NMR binding curve is interrupted by sharp increases in linewidth in the concentration range between 0.1 mM and 10 mM  $\text{Cl}^-$ . Fig. 9 shows representative spectra for PS II membranes. As indicated by the measurements of the excess linewidth at each  $[\text{Cl}^-]$  concentration, the size of  $\Delta\nu_t$  alternately decreases and increases several times as the  $[\text{Cl}^-]$  increases. The results for two individual preparations are plotted in Fig. 10 (thylakoids) and Fig. 11 (PS II membranes).

Fig. 12 shows the  $^{35}\text{Cl}$ -NMR binding curve obtained by averaging the results of a large number of linewidth measurements on a number of different thylakoid preparations (see legend of Fig. 12 for details). It is clear from these measurements that the excess linewidth as a function of  $[\text{Cl}^-]$  does not give a smoothly descending curve. Although, as expected,  $\Delta\nu_t$  decreases sharply in the concentration range where the OEC is activated (near the  $K_A'$  for  $\text{Cl}^-$ ), the curve is interrupted by increases in linewidth at 0.3 mM, 0.75 mM, 3.25 mM, and 7.0 mM  $\text{Cl}^-$ . As indicated in Fig. 5, these  $^{35}\text{Cl}$ -NMR linewidth maxima occur at roughly the same  $[\text{Cl}^-]$  as the deviations in the  $\text{Cl}^-$  activation curve for the Hill activity.

Figure 9.  $^{35}\text{Cl}$ -NMR spectra of PS II membranes at various  $\text{Cl}^-$  concentrations. The Chl concentration for these and other NMR measurements of PS II membranes was  $0.5 \text{ mg ml}^{-1}$ . The top spectrum in each column represents a  $\text{Cl}^-$  buffer standard (10 mM NaCl in 400 mM sucrose, 20 mM MES, pH 6.0, 1,000 accumulations). The added line-broadening was 15 Hz for the left column and 10 Hz for the right column. For the PS II membranes, the number of accumulations ranged from 24,000 ( $0.2 \text{ mM Cl}^-$ ) to 2,000 ( $7.0 \text{ mM Cl}^-$ ).

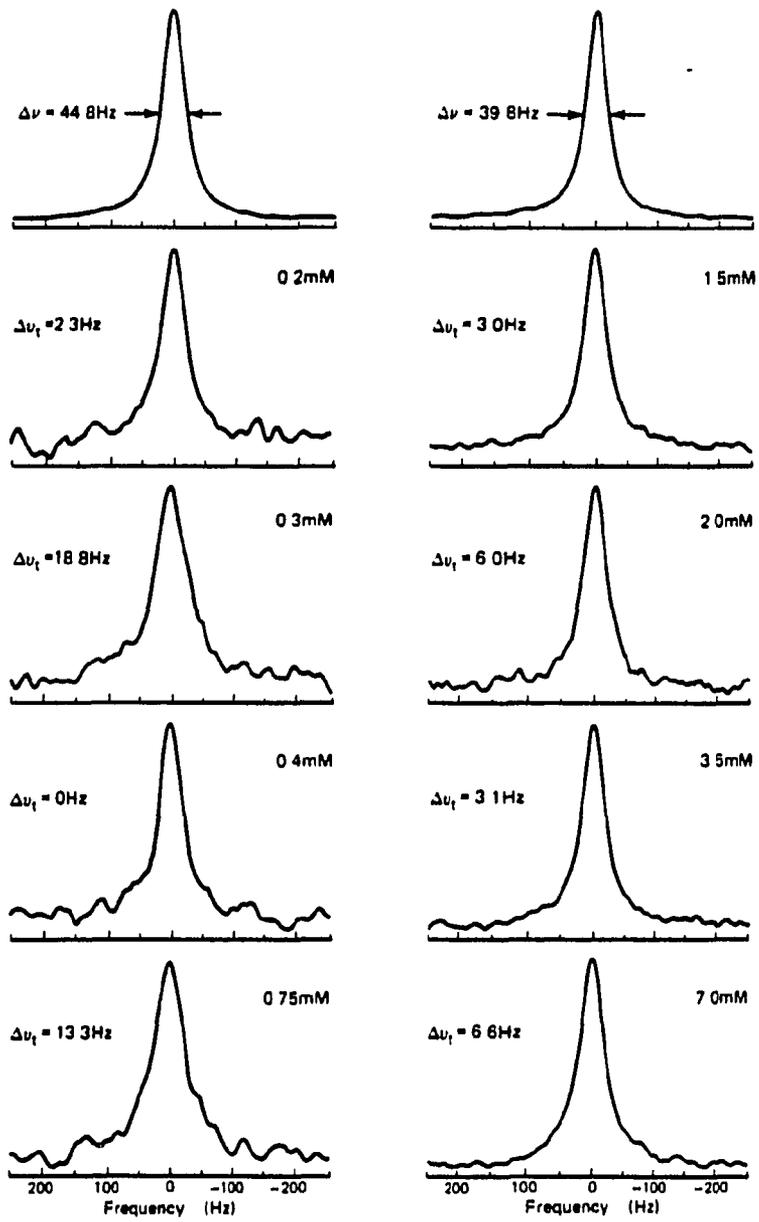


Figure 10.  $^{35}\text{Cl}$ -NMR binding curve for thylakoids (excess linewidth versus  $[\text{NaCl}]$ ). Data points for linewidth measurements on two preparations are plotted. The Chl concentration for these and other NMR measurements of thylakoids was  $1.0 \text{ mg ml}^{-1}$ . The pH was 7.2.

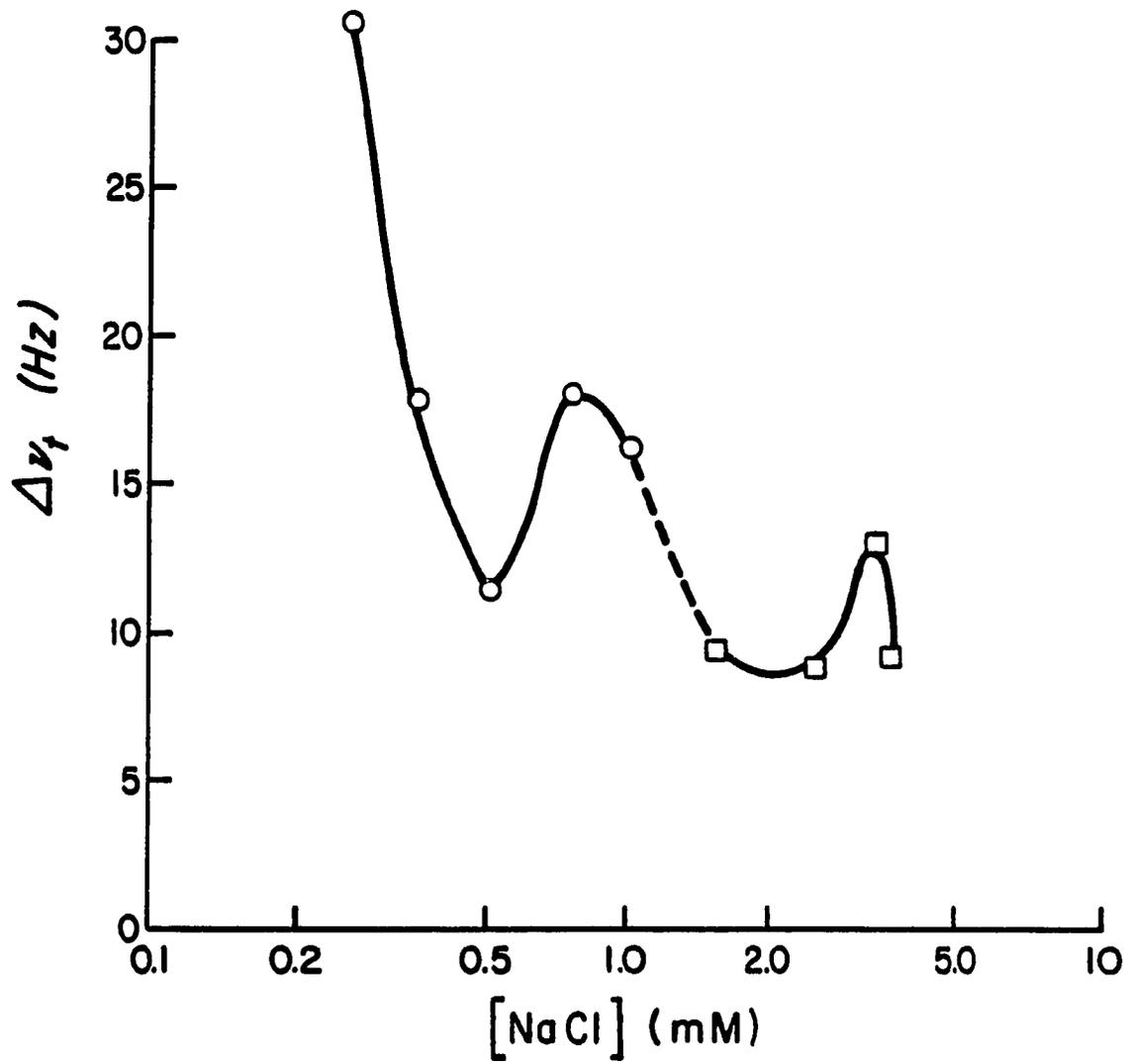


Figure 11.  $^{35}\text{Cl}$ -NMR binding curve for PS II membranes (excess linewidth versus  $[\text{NaCl}]$ ). Data points for linewidth measurements on two preparations are plotted. The pH was 6.0.

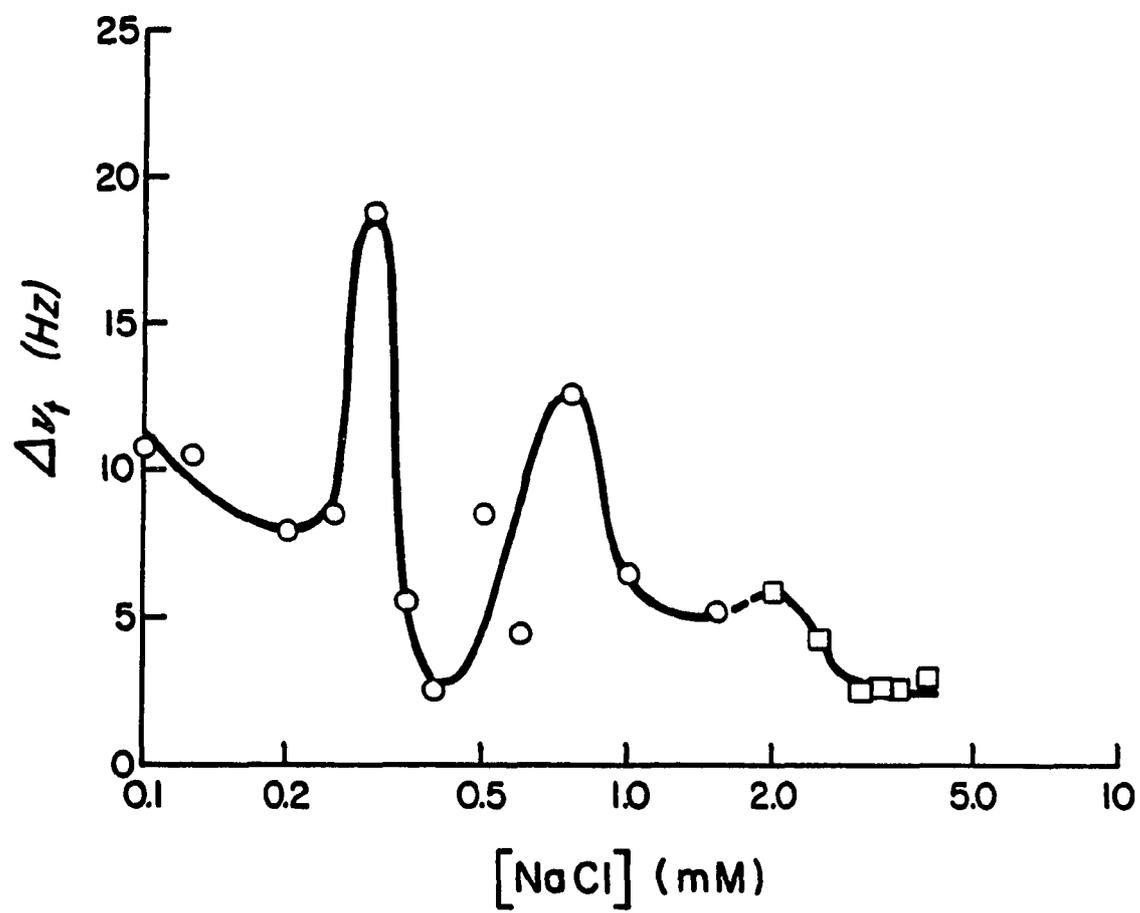
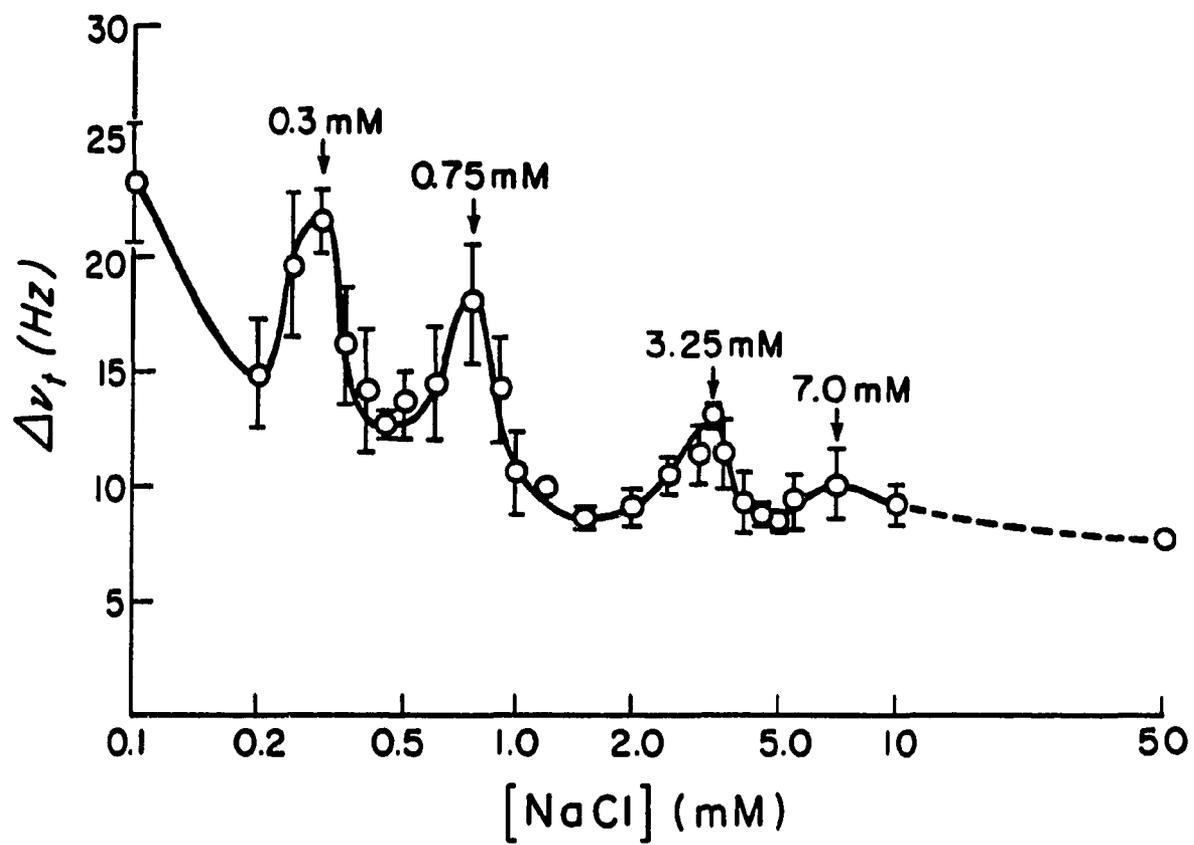


Figure 12.  $^{35}\text{Cl}$ -NMR binding curve for thylakoids. The mean Hill activity for all of the preparations (34 in all) was  $343 \mu\text{mol DCIP red. (mg Chl)}^{-1} \text{ hr}^{-1}$ . The Chl concentration was  $1.0 \text{ mg ml}^{-1}$ . Each point with an error bar is the mean value for  $\Delta\nu_t$  at a given  $[\text{Cl}^-]$ , and represents the mean of 3-8 measurements on different thylakoid preparations. The error bars represent the sample standard error. Since the values of  $\Delta\nu_t$  were not normalized, they include systematic variation in  $\Delta\nu_t$  from several sources: 1) slight variation in the concentration of endogenous  $\text{Cl}^-$ , which tends to shift the entire curve to the right or left; 2) inaccuracies in the measurement of  $\Delta\nu_f$  (i.e. the  $\text{Cl}^-$  buffer standard), which shifts the entire curve up or down; and 3) variation in the activity of the preparations, which affects the magnitude of the linewidth maxima (see Fig. 14). Approximately 400 hours of NMR instrument time (for both accumulating and processing) were required to obtain the complete curve.



A similar phenomenon is observed in PS II membranes (Fig. 13). In this system, the overall linewidths are slightly narrower, probably as a result of the removal of the non-specific  $\text{Cl}^-$  binding sites present in thylakoids (Balanu et al., 1984). In these preparations, the linewidth maxima appear at 0.3 mM, 0.75 mM, 2.0 mM and 7.0 mM  $\text{Cl}^-$ . The reason for the apparent shift of the third maximum to lower  $[\text{Cl}^-]$  is not known. The ratio of the linewidths for the four linewidth maxima is approximately 5:3:2:1-2.

After measuring the  $^{35}\text{Cl}$ -NMR linewidths on a number of different spinach preparations during the course of this study, it became apparent that the measured linewidths for each of these linewidth maxima depends strongly on the activity of the preparation. Moreover, each of the linewidth maxima shows a slightly different dependence on the enzyme activity. The results for PS II membranes are plotted in Fig. 14. Because of this trend, only the  $^{35}\text{Cl}$ -NMR results from preparations with the highest activity have been used.

In order to verify that these NMR results did not arise simply from an instrumental artifact, we measured a  $^{35}\text{Cl}$ -NMR binding curve for a dilute solution of bovine serum albumin, using the same buffer as for the thylakoids and identical instrument settings (Fig. 15). For this protein, which contains a number of  $\text{Cl}^-$  binding sites, we found that  $\Delta\nu_t$  shows little significant deviation from a linear dependence against  $\log [\text{Cl}^-]$ . Previous  $^{35}\text{Cl}$ -NMR studies of serum albumin at higher  $[\text{Cl}^-]$  have also failed to show any deviation from expected behavior (Norne et al., 1975).

Since  $\text{Br}^-$  has been shown to activate  $\text{O}_2$ -evolution nearly as effectively as  $\text{Cl}^-$  (Kelley and Izawa, 1978; Critchley et al., 1982), but is not detected by NMR at the frequency used here, we tested whether the

Figure 13.  $^{35}\text{Cl}$ -NMR binding curve for PS II membranes. The mean activity for all the preparations (10 in all) was  $383 \mu\text{mol O}_2 (\text{mg Chl})^{-1} \text{hr}^{-1}$ . The Chl concentration was  $0.5 \text{ mg ml}^{-1}$ .

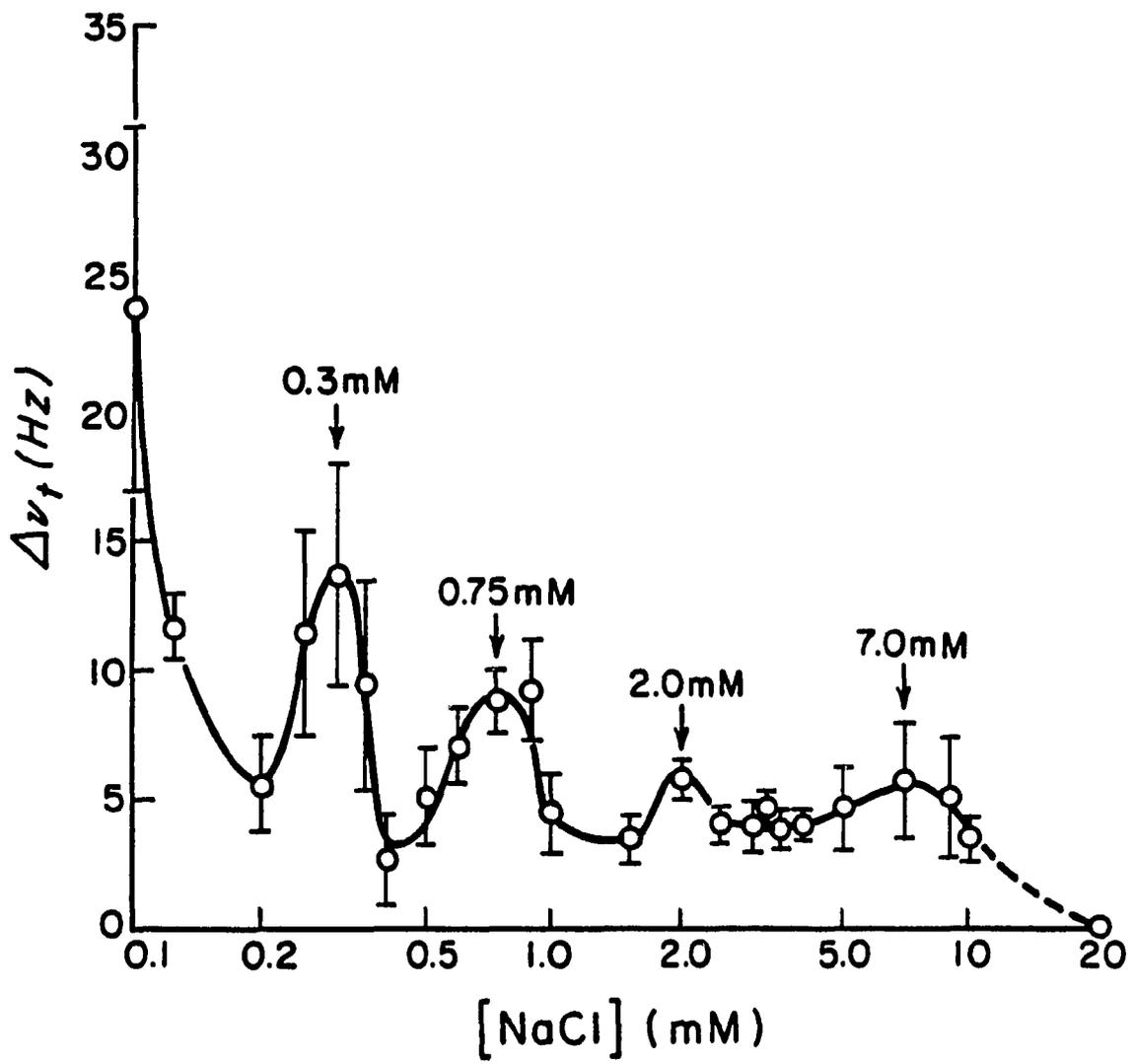


Figure 14. Dependence of the  $^{35}\text{Cl}$ -NMR linewidth maxima on the OEC activity for PS II membranes at four different  $[\text{Cl}^-]$ .

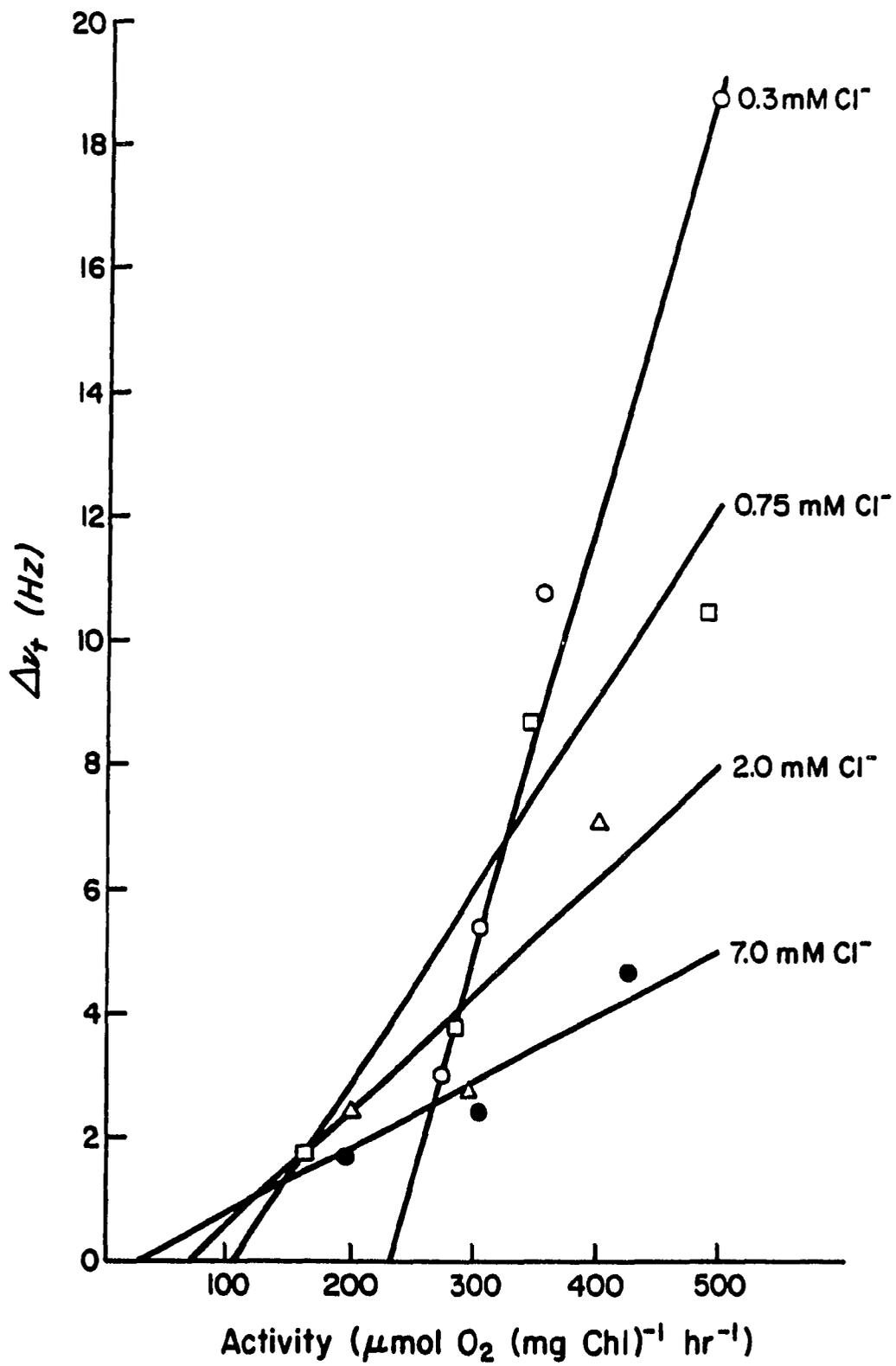
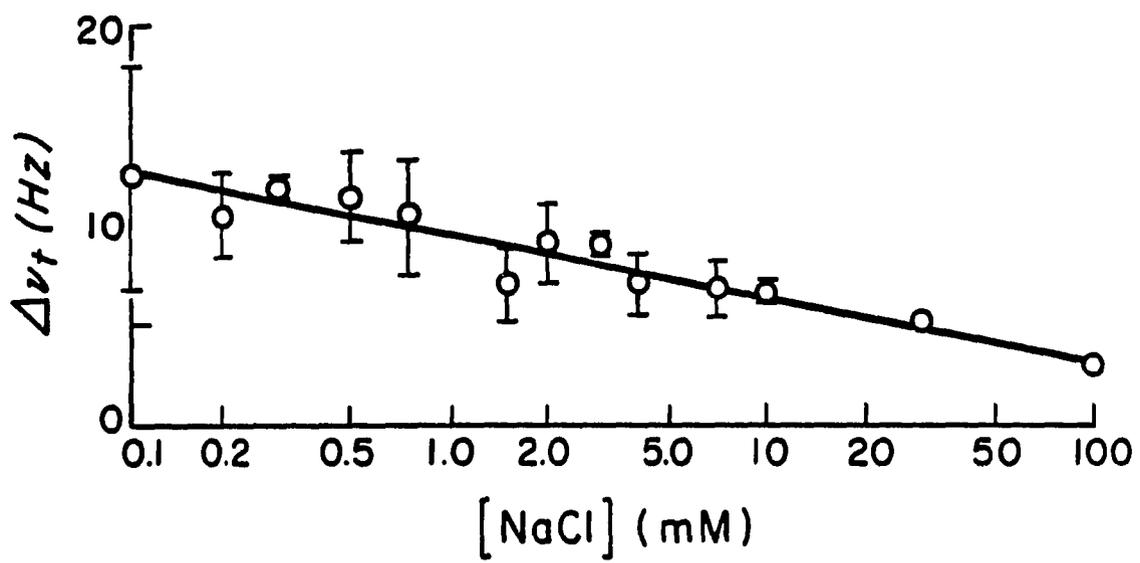


Figure 15.  $^{35}\text{Cl}$ -NMR binding curve for bovine serum albumin. The protein was dissolved at a concentration of  $0.1 \text{ mg ml}^{-1}$  in 50 mM HEPES at pH 7.2. Error bars show the sample standard error for 2-4 measurements of  $\Delta\nu_t$ .



excess line-broadening we have observed in PS II can be reduced by addition of a small, constant amount of  $\text{Br}^-$  (0.1 mM) to the PS II suspension. The resulting  $^{35}\text{Cl}$ -NMR binding curve (Fig. 16), shows an overall decrease in excess linewidth and a substantial loss of line-broadening at the lowest  $[\text{Cl}^-]$ . In addition, the linewidth maxima are diminished by about 40%, with the maximum at 0.3 mM being shifted upward to 0.35 mM and the maximum at 0.75 mM shifted downward to 0.6 mM.

Since Homann (1985) has demonstrated that the binding of  $\text{Cl}^-$  to PS II membranes requires the protonation of a group having a  $\text{pK}_a$  of approximately 6.0, we examined the pH dependence of  $\Delta\nu_t$  at one of the linewidth maxima (0.75 mM  $\text{Cl}^-$ ) to determine whether there is any correlation between the pH dependence for  $\text{Cl}^-$  binding and the pH dependence for  $\text{Cl}^-$  activation of  $\text{O}_2$ -evolution. Fig. 17 shows that the pH optimum for  $\text{Cl}^-$  binding at this  $[\text{Cl}^-]$  is also at 6.0. Interestingly, however, there are also two smaller peaks at approximately 5.4 and 6.5. Between these three peaks, the linewidth becomes very narrow, reaching a minimum at pH 5.7 and 6.2. A third narrowing of the linewidth occurs at pH 7.6.

#### D. Discussion

The results presented here demonstrate that it is possible to measure  $\text{Cl}^-$  binding to the OEC in spinach thylakoids and PS II membranes by  $^{35}\text{Cl}$ -NMR. However, the appearance of linewidth maxima in the  $^{35}\text{Cl}$ -NMR binding curve (Figures 12 and 13) cannot be adequately explained by equations 1 and 2, which assume that  $\Delta\nu_b$  is a constant and that  $f_b$  decreases monotonically with increasing  $[\text{Cl}^-]$ . The only meaningful way to explain these sharp,  $\text{Cl}^-$  dependent increases in linewidth is to propose that the additional line-broadening arises from  $\text{Cl}^-$  binding to

Figure 16.  $^{35}\text{Cl}$ -NMR binding curve for PS II membranes in the presence of 0.1 mM NaBr. The mean value for the activity was  $332 \mu\text{mol O}_2 (\text{mg Chl})^{-1} \text{ hr}^{-1}$ . The dashed line shows the curve from Fig. 13. Error bars show the sample standard error for 2-3 measurements of  $\Delta\mathcal{V}_t$ .

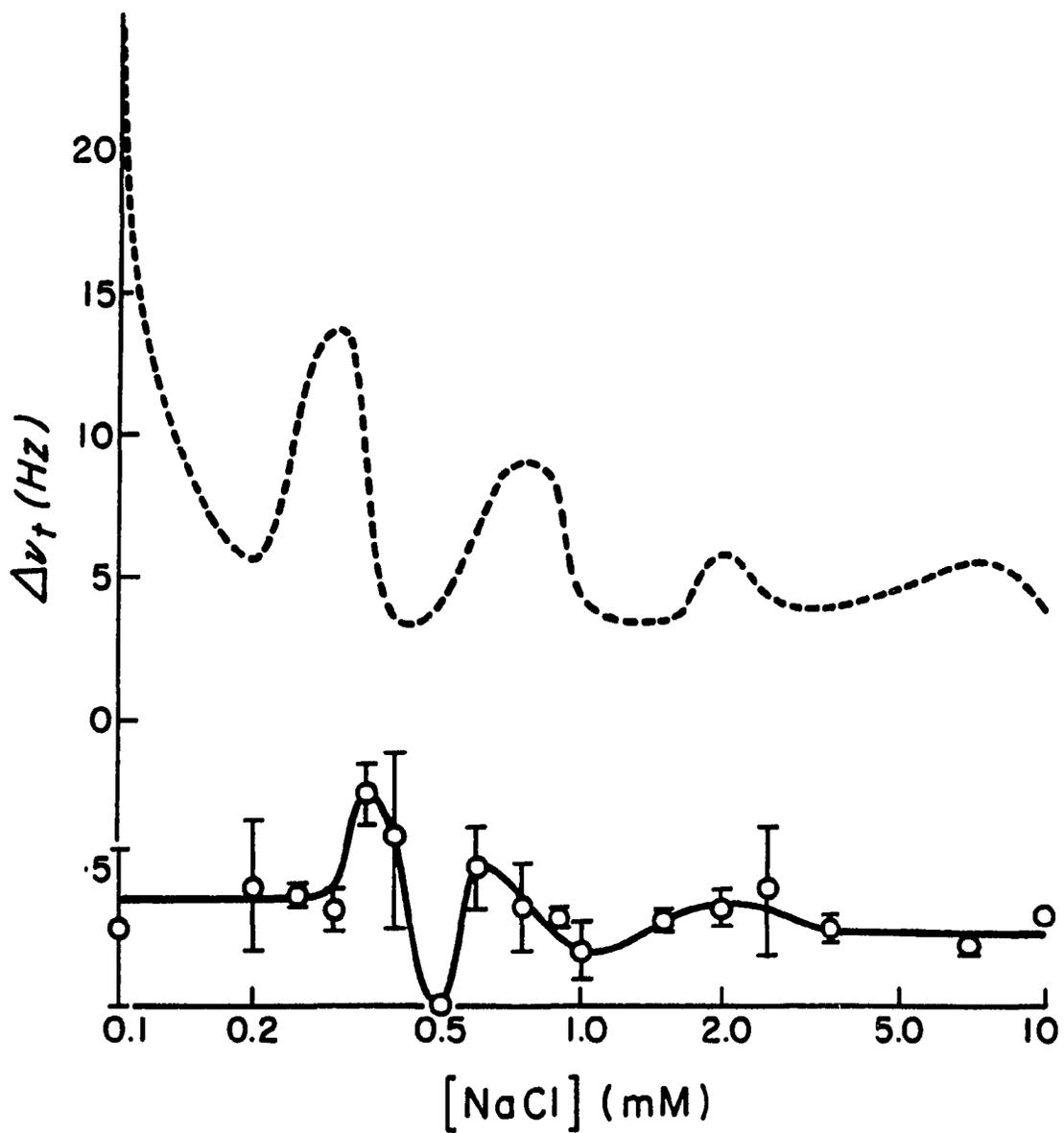
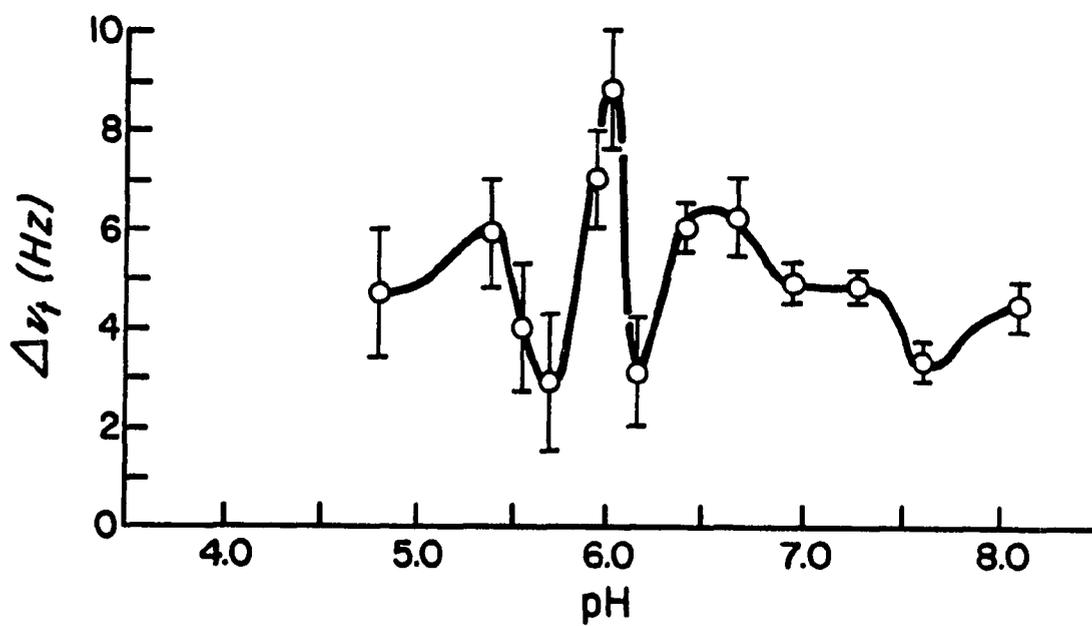


Figure 17. pH dependence of  $\Delta\mathcal{V}_t$  at 0.75 mM  $\text{Cl}^-$ . The mean activity of the preparations (7 in all) was  $338 \mu\text{mol O}_2 (\text{mg Chl})^{-1} \text{hr}^{-1}$  at pH 6.0. Error bars show the sample standard error for 3-7 measurements of  $\Delta\mathcal{V}_t$ . The buffer concentration was 20 mM. In order to precisely control the pH, buffers appropriate for each pH range were used: Tricine at pH 8.08, HEPES at pH 6.65-7.59, MES at pH 5.69-6.39, and citrate/phosphate at pH 4.79-5.54. All pH values represent the final (measured) pH after diluting the concentrated PS II sample (in MES buffer at pH 6.0) by 5-fold with the given buffer.



previously non-exchanging sites within the OEC, and that these sites are opened up to exchange by the addition of  $\text{Cl}^-$ . This explanation implies that  $\text{Cl}^-$  binding is at least partially cooperative, and that conformational changes in the OEC are involved. The observation of complex kinetic cooperativity with respect to  $\text{Cl}^-$  in the steady-state kinetics of activation (Figures 3-5) lends support to this interpretation. Moreover, both measurements indicate that four distinct  $\text{Cl}^-$  binding sites (at approximate concentrations of 0.25-0.3 mM, 0.75-1.0 mM, 3.25-3.5 mM, and 6.0-7.0 mM  $\text{Cl}^-$  in thylakoids) contribute to these effects. The relative contribution of each site to the excess linewidth is approximately 5:3:2:1-2. Although equations have been devised to explain simple cooperativity in enzyme kinetics (Segel, 1975), there are as yet no equations to explain complex cooperativity in NMR binding studies of proteins.

The  $^{35}\text{Cl}$ -NMR linewidth maxima observed here are not simply the result of a non-specific ionic strength effect. It will be shown in Chapter 4 that these same linewidth maxima appear in NaCl-washed PS II membranes in the presence of 2.0 mM  $\text{CaSO}_4$ .

In an earlier  $^{35}\text{Cl}$ -NMR study of  $\text{Cl}^-$  binding to thylakoids from halophytes, Bajanu et al. (1984) did not observe any anomalies in a plot of  $\Delta\nu_c$  vs.  $[\text{Cl}^-]$  at a Chl concentration of 2.9 mg ml $^{-1}$  for Avicennia germinans. However, since the  $\text{Cl}^-$  requirement of the halophyte thylakoids is much higher than in spinach (Critchley et al., 1982), it is possible that the  $\text{Cl}^-$  binding mechanism is also different. In addition, the NMR measurements of the halophytes were done at  $\text{Cl}^-$  concentrations 10- to 1,000-fold higher than those employed here.

Although to our knowledge there are no other reports of sharp increases in  $^{35}\text{Cl}$ -NMR line-broadening in a protein system, an equivalent

phenomenon has been reported for counter-ion binding to a synthetic polymer. By observing the binding of another quadrupolar nucleus,  $^{23}\text{Na}$ , which binds to the polyanion polymethacrylic acid (PMA), Gustavsson et al. (1976, 1978; see also Lindman, 1978) detected a large increase in the  $^{23}\text{Na}$  relaxation rate as negative charges on the PMA were progressively neutralized with HCl. They attributed this effect to a cooperative conformational transition in the PMA molecule, transforming it from a compact globular form to an expanded coil. The phenomenon of  $\text{Cl}^-$  binding to protonatable groups within the OEC (Homann et al., 1983) may involve similar changes in conformation.

There is already substantial evidence to indicate that bound  $\text{Cl}^-$  in the OEC is tightly associated with a sequestered pool of protons (Theg and Homann, 1982; Theg et al., 1982; Homann et al., 1983). Some of these protons are believed to be pumped into the sequestered domains by the light-driven oxidation of water (Theg et al., 1982). Since the release of  $\text{Cl}^-$  from the membrane requires the removal of these protons by treatment at high pH and the avoidance of illumination, it is reasonable to expect that the uptake of  $\text{Cl}^-$  by the OEC requires the reverse reaction, i.e., the protonation of these same groups. If this protonation can be accomplished (albeit at fairly low efficiency in the absence of  $\text{Cl}^-$ ) by brief illumination of the thylakoids (Theg and Homann, 1982) then this would explain why pre-illumination partially eliminates the inflections in the  $\text{Cl}^-$  activation curve (compare Figures 7 and 8 with 4 and 5). The likely reason, as Homann (1985) has suggested, is that  $\text{H}^+$  must bind to sites on the OEC before  $\text{Cl}^-$  binding may proceed. The binding of  $\text{Cl}^-$  is thus limited by the concentration of  $\text{H}^+$ , its co-activator (see Kurganov, 1982).

At low light intensity, the production of  $H^+$  by the  $Cl^-$  depleted OEC is apparently slow enough to limit the binding of  $Cl^-$  at each of the binding sites. The result is that inflections appear in the activation curve as each site is titrated with  $Cl^-$ . At high light intensity, the sites are saturated with  $H^+$ , and consequently the cooperativity with respect to the  $[Cl^-]$  vanishes (Fig. 3; Kelley and Izawa, 1978). A similar effect has been observed in CTP synthetase from E. coli, which exhibits diminished cooperativity with respect to the concentration of substrate (glutamine) as the concentration of GTP (an allosteric activator) is raised (Levitzki and Koshland, 1969).

Studies of  $Cl^-$  uptake by other  $Cl^-$  binding proteins have indicated that in some cases Cl is capable of binding to sites that become exposed as the pH is lowered (Saroff, 1959; Loeb and Saroff, 1964; Chiancone et al., 1975; see also Chapter 1). This phenomenon has been attributed to the existence of salt-bridges within these proteins. When the carboxylate partner of a positively-charged residue is neutralized, the latter group may become available to bind  $Cl^-$  (Saroff, 1959). An analogous situation may occur in the OEC. For example, the four linewidth maxima that we have observed in thylakoids and PS II membranes may reflect the existence of four sets of such salt-bridges. If these four domains contain an unequal number of salt-bridges (distributed in the proportion 5:3:2:1-2), then this would explain both the relative sizes of the linewidth maxima and the relative magnitude of each increase in  $O_2$ -evolution between plateaus in the  $Cl^-$  activation curve. This hypothesis predicts that there are at least 11-12 sequestered  $Cl^-$  binding sites within the OEC. The results of the  $Br^-$  experiments indicate that  $Br^-$  competes with  $Cl^-$  at these sites.

The pH dependence of  $Cl^-$  binding exhibits a maximum excess

linewidth at pH 6.0, a value which agrees exactly with Homann's determination of the  $pK_a$  from kinetic measurements (Homann, 1985). However, unlike the pH-activity curve for  $O_2$ -evolution, which is a smooth bell-shape (Critchley, 1985), the linewidth vs. pH curve has two minima and two smaller maxima flanking the main peak at pH 6.0. Although we agree with Homann's assignment of this site to a histidyl residue, we believe that a cluster of three histidines provides the best explanation of both the activation of  $O_2$ -evolution by  $Cl^-$  and the three peaks that we observe in the pH dependence of the  $^{35}Cl$ -NMR linewidth. A cluster of residues that is capable of being rapidly protonated and deprotonated in this pH region could control the movement of  $Cl^-$  and  $H^+$  into the sequestered domains outlined above. This model is discussed in greater detail in Chapter 5.

#### **E. Summary**

Experiments presented in this chapter demonstrate that  $Cl^-$  binding to the OEC can be monitored by  $^{35}Cl$ -NMR.  $Cl^-$  binding depends strongly on  $H^+$ , and can be partially eliminated by competition with  $Br^-$ . The  $Cl^-/H^+$  binding mechanism appears to involve cooperative conformational changes within four sequestered domains of the OEC.

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### III. THE EFFECT OF CHLORIDE ON THE THERMAL INACTIVATION OF OXYGEN EVOLUTION

#### A. Introduction

Mild heat treatment of thylakoids and PS II membranes has been used for many years as both a convenient means to inactivate the water-splitting reactions and to study the structure of the OEC (Katoh and San Pietro, 1967; Yamashita and Butler, 1968; Lozier et al., 1971; Nash et al., 1985). Most studies have focused on the stability of a pool of bound manganese that is believed to be directly involved in the light-driven oxidation of water, since this Mn is released by heating (Cheniae and Martin, 1966; Lozier et al., 1971; Kimimura and Katoh, 1972; Wydrzynski and Sauer, 1980; Cramer et al., 1981). Detachment of catalytic Mn from its binding site has been proposed as the chief reason for the irreversible loss of enzyme activity in thylakoids (Kimimura and Katoh, 1972).

The available evidence for a direct correlation between Mn release and inhibition of the Hill reaction in thylakoids is rather contradictory. Kimimura and Katoh (1972), using Euglena chloroplasts, and Cramer et al. (1981), using spinach chloroplasts, both reported that Mn release occurs in the same temperature range as the loss of Hill activity. Cheniae and Martin (1966), however, found that in chloroplast particles from Scenedesmus the rate of inactivation of the Hill reaction at 50°C was much faster than the rate of Mn loss.

A new perspective to this problem was provided by the discovery that Cl<sup>-</sup> depletion dramatically alters the sensitivity of O<sub>2</sub>-evolution to thermal inactivation. Coleman et al. (1984) and Nash et al. (1985) found that Cl<sup>-</sup> depletion increases the level of inhibition at a given

heating temperature. The protective effect of  $\text{Cl}^-$  indicates that it might play a role in stabilizing the structure of the OEC. In view of the fact that a highly-ordered structure appears to be involved in  $\text{Cl}^-$  binding to the native system (see discussion in Chapter 2), we decided to examine the effect of heating on both  $\text{Cl}^-$  binding (by  $^{35}\text{Cl}$ -NMR) and  $\text{Cl}^-$  activation of  $\text{O}_2$ -evolution in EDTA-washed preparations, and to relate these effects to the stability of the catalytic Mn.

## **B. Materials and Methods**

Sample Preparation. Procedures for preparing and assaying  $\text{Cl}^-$  depleted thylakoids and PS II membranes are described in Chapter 2. Partially  $\text{Cl}^-$  depleted thylakoids were prepared as described, except that the washed thylakoids were simply isolated and washed in  $\text{Cl}^-$  free buffer, and were not subjected to high-pH/Gramicidin treatment. This procedure produced a level of  $\text{Cl}^-$  depletion (as measured by the Hill activity  $\pm \text{Cl}$ ) of about 35%.

Heating Methods. For heating large numbers of small aliquots (experiments in Figs. 18, 21 and 22), glass tissue grinders (Kontes Glass) were used to spread the sample into a very thin, uniform layer between the pestle and the inner wall of the holding tube. For larger aliquots, the thylakoids or PS II membranes were spread in a thin layer at the bottom of a large glass beaker. Because of the slightly greater thickness of the layer in the latter set-up, the level of inhibition produced by the heat treatment was slightly less. Heating vessels were allowed to equilibrate to the bath temperature before membrane suspensions were added. This was done to insure uniformity of treatment at equilibrium conditions. The vessels containing the membrane

suspensions were heated in a water bath-circulator (Lauda-Brinkmann RC-3) with a stability of  $\pm 0.1^{\circ}\text{C}$ . The temperature was continually monitored by a Solomat T135 digital thermometer. Aliquots to be heated were first removed from the ice and allowed to warm to room temperature for 5 min before being placed in the water bath for 3 min. All treatments were done in darkness. Heated aliquots were immediately returned to the ice.

Manganese Release. For the EPR measurements of Mn release from thylakoids, the following procedure was used. A small aliquot of thylakoids ( $780 \mu\text{l}$  at  $3.5 \text{ mg Chl ml}^{-1}$ ) in buffer consisting of  $0.2 \text{ mM}$  EDTA and  $50 \text{ mM}$  HEPES (pH 7.2) was mixed with  $20 \mu\text{l}$  of  $2 \text{ M}$  NaCl/buffer ( $50 \text{ mM}$   $\text{Cl}^{-}$  final concentration) or buffer without  $\text{Cl}^{-}$ , and allowed to incubate for 5 min at room temperature. A small amount of EDTA was included in the buffer in order to ensure that any Mn that was released from the OEC did not re-bind to non-specific sites on the membrane (Khanna et al., 1983). After heating for 3 min, the sample was quenched on ice for 10 min. A small aliquot was removed for the Hill reaction assay, and the remainder was centrifuged for 6 min at  $9,000 \times g$ . The pellet was rinsed and resuspended in buffer, and the Chl concentration was measured again for each sample. Immediately prior to EPR measurement, each sample was treated with HCl at a final concentration of  $0.1 \text{ N}$  in order to release all of the bound Mn. Additional HCl did not increase the signal amplitude.

EPR Measurements. The hexaquo- $\text{Mn}^{2+}$  concentration was measured at room temperature by the EPR method (see Yocum et al., 1981; Khanna et al., 1983; Miller and Cox, 1984). A set of  $\text{Mn}^{2+}$  standards in buffer ( $0.5$  to  $4.0 \mu\text{g Mn ml}^{-1}$ ) was prepared by serial dilution of a concentrated atomic absorption standard solution (Spex, Inc., Metuchen, N.J.). These

solutions were used to generate a standard curve to calibrate the peak-to-trough height of the 2nd low-field line of the Mn EPR signal. EPR spectra were obtained at X-band using a quartz aqueous TM flat cell. The spectrometer was a Varian E-line instrument equipped with a Varian TM cavity. Spectrometer settings were as follows: scan range:  $\pm 500$  G, field set: 3300 G, modulation frequency: 100 kHz, modulation amplitude: 10 G, time constant: 0.064 s, scan time: 4 min, power: 80 mW, frequency: 9.413 GHz.

Unheated thylakoids were found to contain an average of 5.5 Mn per 400 Chl. This is within the range of 4-6 Mn per 400 Chl commonly reported for EDTA-washed thylakoids (Yocum et al., 1981; Mansfield and Barber, 1982).

### C. Results

Fig. 18 shows that progressive removal of  $\text{Cl}^-$  from thylakoid membranes has a dramatic effect on the temperature sensitivity of the OEC between 25° and 50°C. In  $\text{Cl}^-$ -sufficient membranes (50 mM  $\text{Cl}^-$ ), the inactivation follows a fairly sharp sigmoid curve with a transition midpoint ( $T_m$ ) of 39.4°C. In partially  $\text{Cl}^-$  depleted membranes, the curve is still sigmoidal, but the  $T_m$  is shifted downward by 2.5°, to 36.9°C. In thoroughly  $\text{Cl}^-$  depleted thylakoids, the  $T_m$  drops to 31.4°C, and the sigmoidicity disappears.

Heating at 38°C for 3 min also affects the steady-state kinetics of  $\text{Cl}^-$  activation of  $\text{O}_2$ -evolution. Fig. 19 shows that this treatment, besides decreasing the activity by 51%, removes all of the plateaus that were observed in the  $\text{Cl}^-$  activation curve in the native system (see Chapter 2, Fig. 4). The elimination of the apparent cooperativity

Figure 18. Effect of  $\text{Cl}^-$  depletion on the thermal inactivation of the Hill reaction in thylakoids. Partially  $\text{Cl}^-$  depleted samples were heated for 3 min in buffer containing 50 mM  $\text{Cl}^-$  ( $\square$ ) or no added  $\text{Cl}^-$  ( $\circ$ ). Thoroughly  $\text{Cl}^-$  depleted samples ( $\bullet$ ) were heated without  $\text{Cl}^-$ . Activity was measured by DCIP reduction in buffer containing 70 mM  $\text{Cl}^-$ . The mean activity after treatment at  $25^\circ\text{C}$  was  $561 \mu\text{mol DCIP reduced (mg Chl)}^{-1} \text{hr}^{-1}$ .

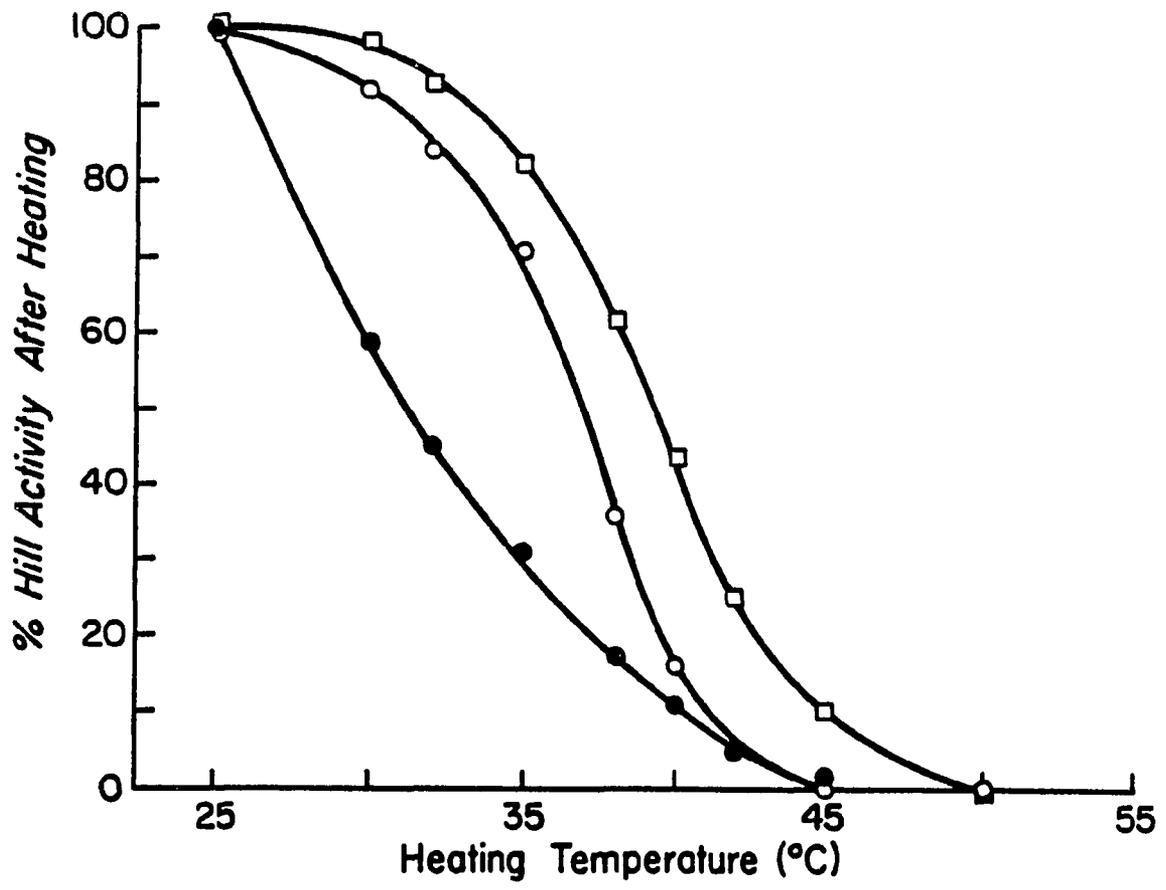
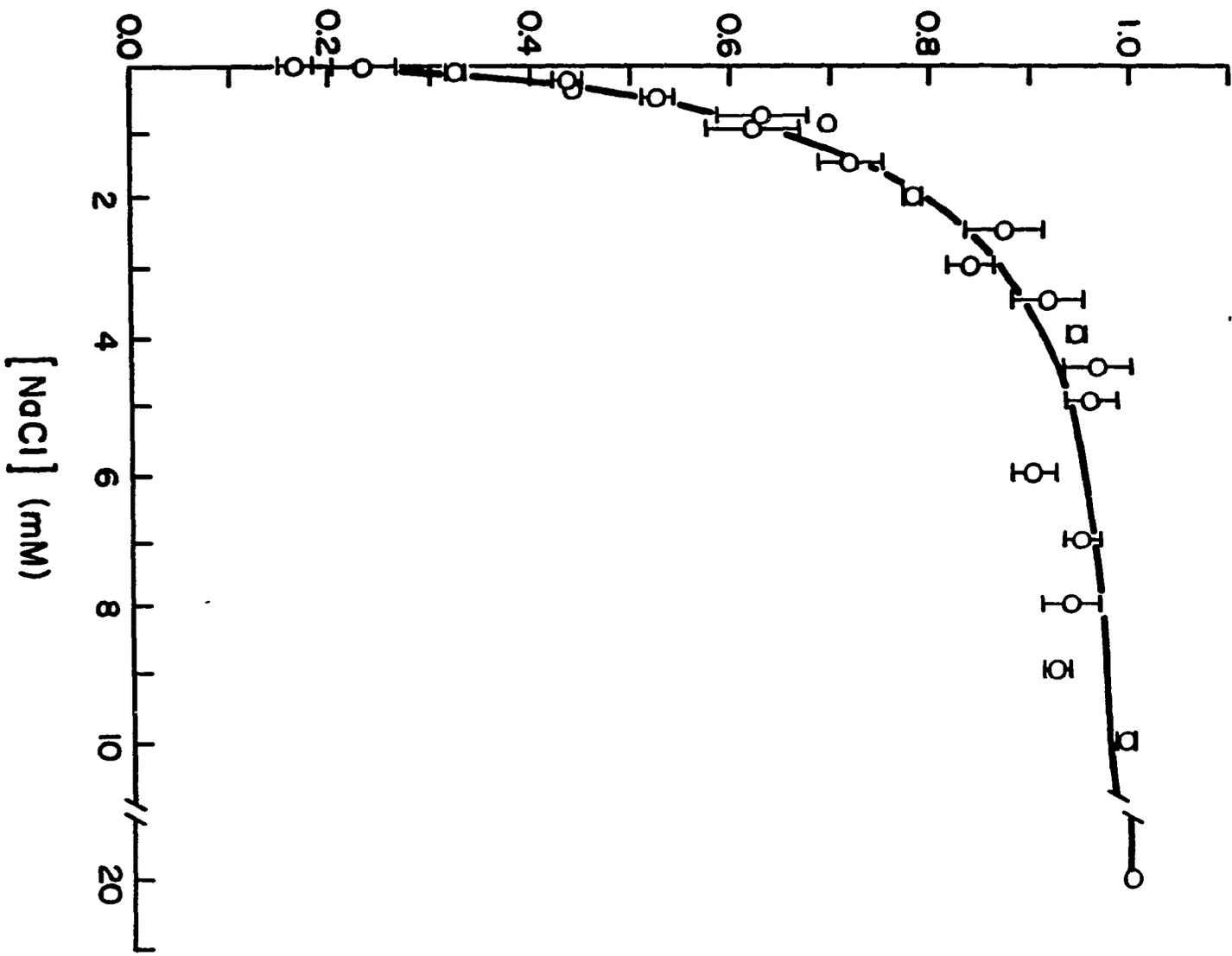


Figure 19.  $\text{Cl}^-$  activation of the Hill reaction in heated thylakoids at 5.5% of saturating light intensity. The thylakoid suspension ( $1.5 \text{ mg Chl ml}^{-1}$ ) was heated for 3 min. at  $38^\circ\text{C}$  without  $\text{Cl}^-$ . The maximum activity (at  $20 \text{ mM Cl}^-$ ) was  $121 \mu\text{mol DCIP reduced (mg Chl)}^{-1} \text{ hr}^{-1}$ . Error bars show the sample standard error for separate measurements on 2-3 different preparations.

*Hill Activity ( $H_2O \rightarrow DCIP$ ) Relative Units*



results in a plot of the relative Hill activity vs.  $\log [Cl^-]$  (Fig. 20) that is a smooth sigmoid. The heated enzyme behaves as though it has a single  $K_A'$  for  $Cl^-$  of about 0.5 mM.

Since previously published reports have suggested that  $Cl^-$  might function as a ligand to the catalytic Mn (Sandusky and Yocum, 1983; Critchley and Sargeson, 1984), we simultaneously examined the effect of partial  $Cl^-$  depletion on the heat release of Mn and the inactivation of the Hill reaction. Figures 21 and 22 show that although there is a significant effect of  $Cl^-$  on the loss of activity between 25° and 35°C, there is no detectable Mn release in this temperature range. In  $Cl^-$ -sufficient samples, Mn release is not correlated with loss of Hill activity until 37% of the activity is already gone. In partially  $Cl^-$  depleted samples, the two processes are not correlated until 62% of the Hill activity is lost.

In  $Cl^-$ -sufficient samples, Hill activity is completely eliminated when 3.0 Mn per PS II have been released. In  $Cl^-$  depleted samples, Hill activity is eliminated when only 2.1 Mn have been released. The amount of Mn released at any given temperature is actually somewhat higher when  $Cl^-$  is present, contrary to what might be expected if  $Cl^-$  were acting as a stabilizing ligand to Mn. Even after treatment at 65°C, only about half of the total Mn is removed from the membrane, in agreement with Nash et al. (1985), although the amount released begins to increase again at 80°C.

In order to determine whether the correlation between increased sensitivity to heating and  $Cl^-$  depletion directly involves the  $Cl^-$  binding sites, we obtained a  $^{35}Cl$ -NMR binding curve for thoroughly  $Cl^-$  depleted thylakoids that had been heated at 38°C. Fig. 23 shows that heat treatment eliminates the linewidth maxima that we had observed in

Figure 20. Relative Hill activity vs.  $\log [Cl^-]$  for heated thylakoids at 5.5% of saturating light intensity. See Fig. 19 for other details.

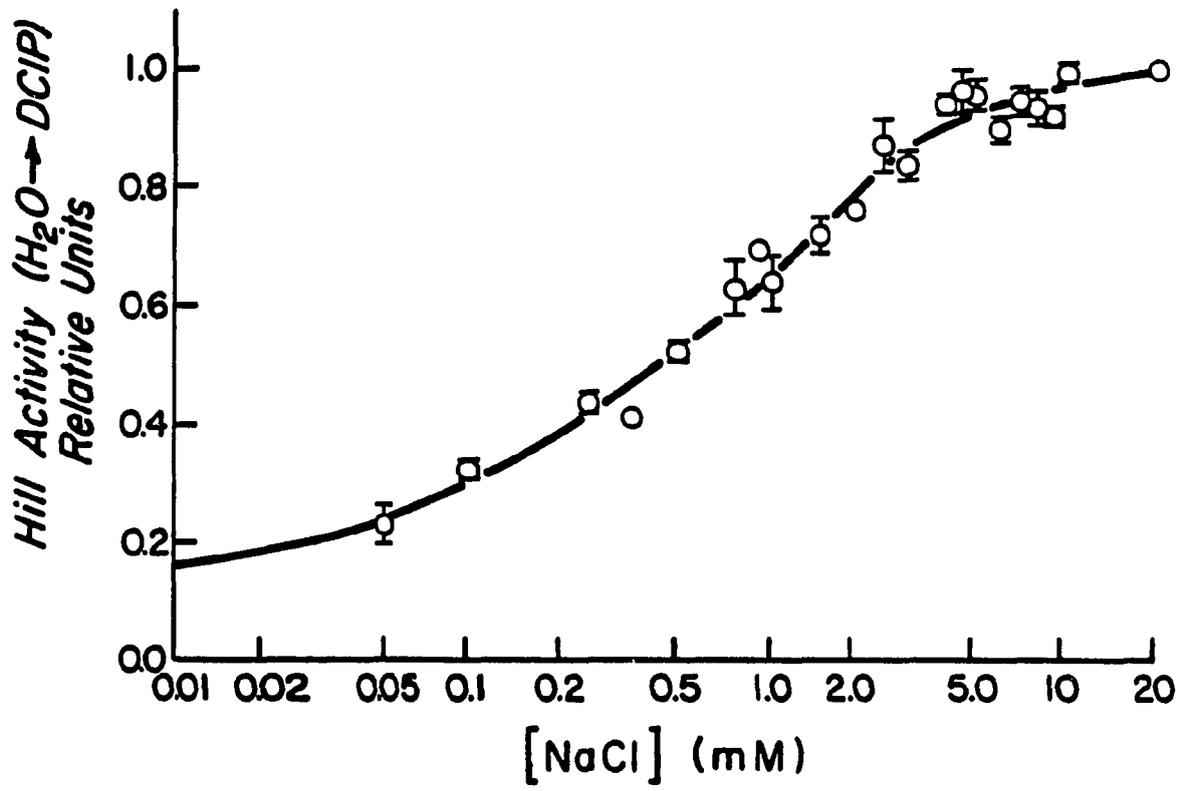


Figure 21. Effect of partial  $\text{Cl}^-$  depletion on the loss of Hill activity and the release of functional Mn in heated thylakoids. Hill activity was measured in 50 mM  $\text{Cl}^-$  after heating for 3 min in the presence (●) or absence (○) of 50 mM  $\text{Cl}^-$ . The mean Hill activity after treatment at 25°C was 369  $\mu\text{mol DCIP reduced (mg Chl)}^{-1} \text{ hr}^{-1}$ . Mn content of the identical thylakoid samples was measured by the EPR method after heating in the presence (■) or absence (□) of 50 mM  $\text{Cl}^-$ . See Materials and Methods for other experimental details.

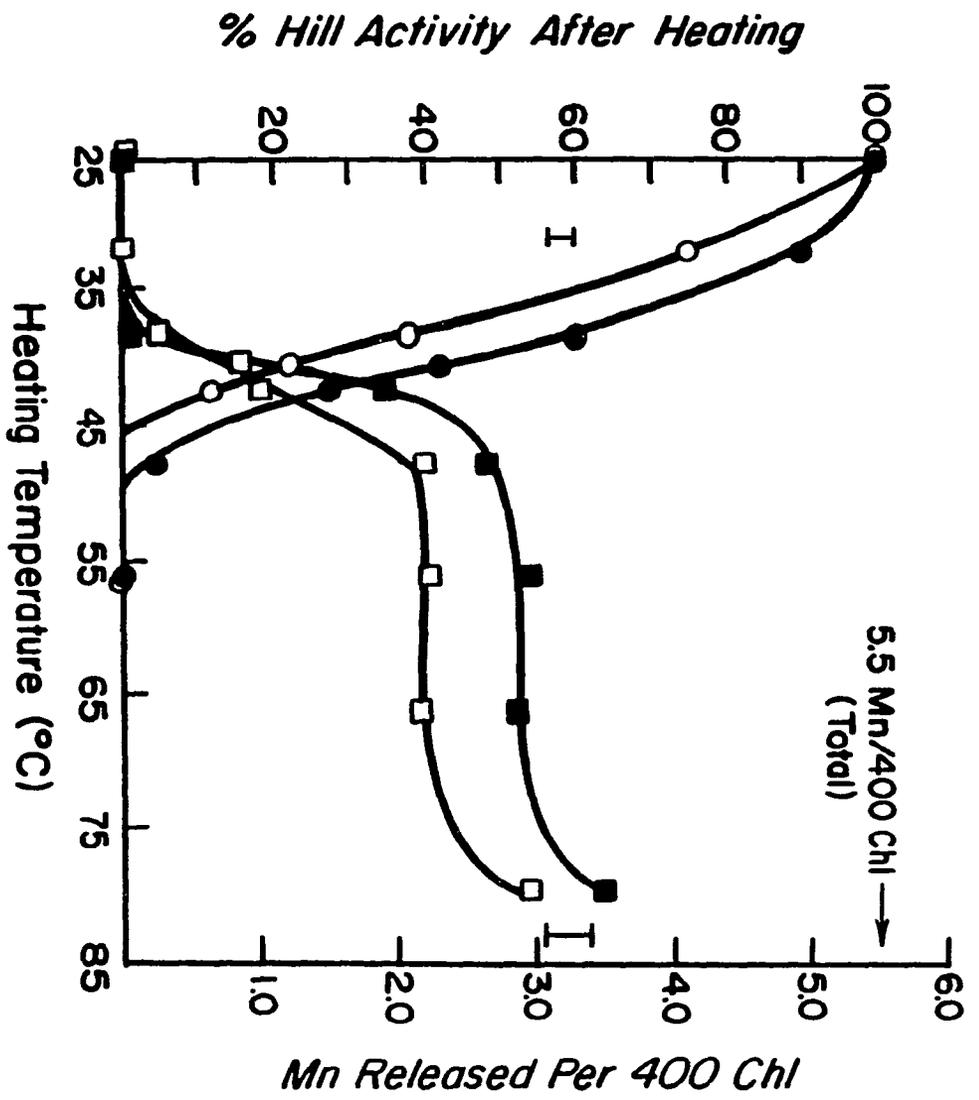


Figure 22. Effect of partial  $\text{Cl}^-$  depletion on the correlation between the loss of Hill activity and the release of functional Mn in heated thylakoids. Data are taken from Fig. 21. (■) heated in 50 mM  $\text{Cl}^-$ ; (□) heated in the absence of  $\text{Cl}^-$ .

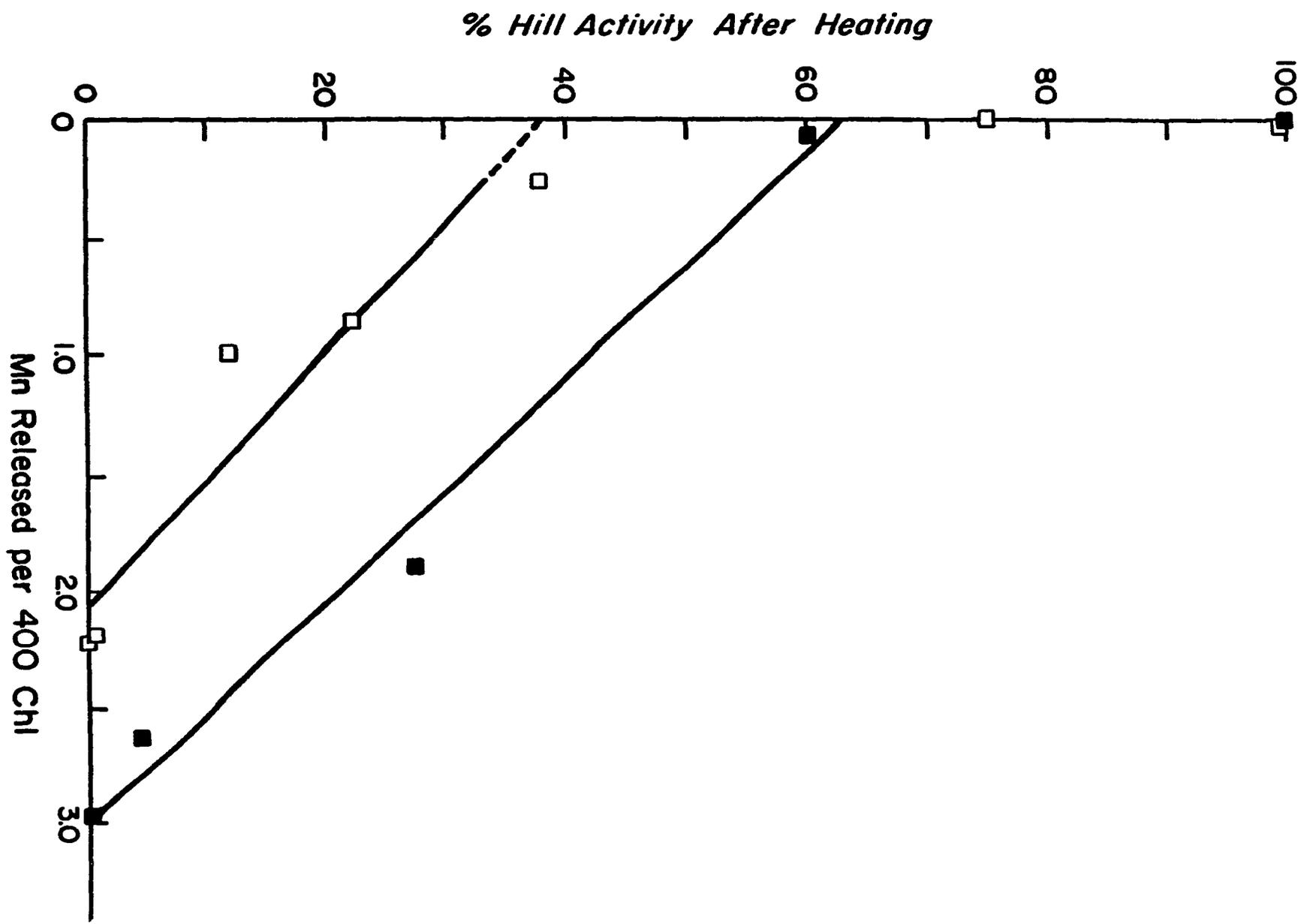
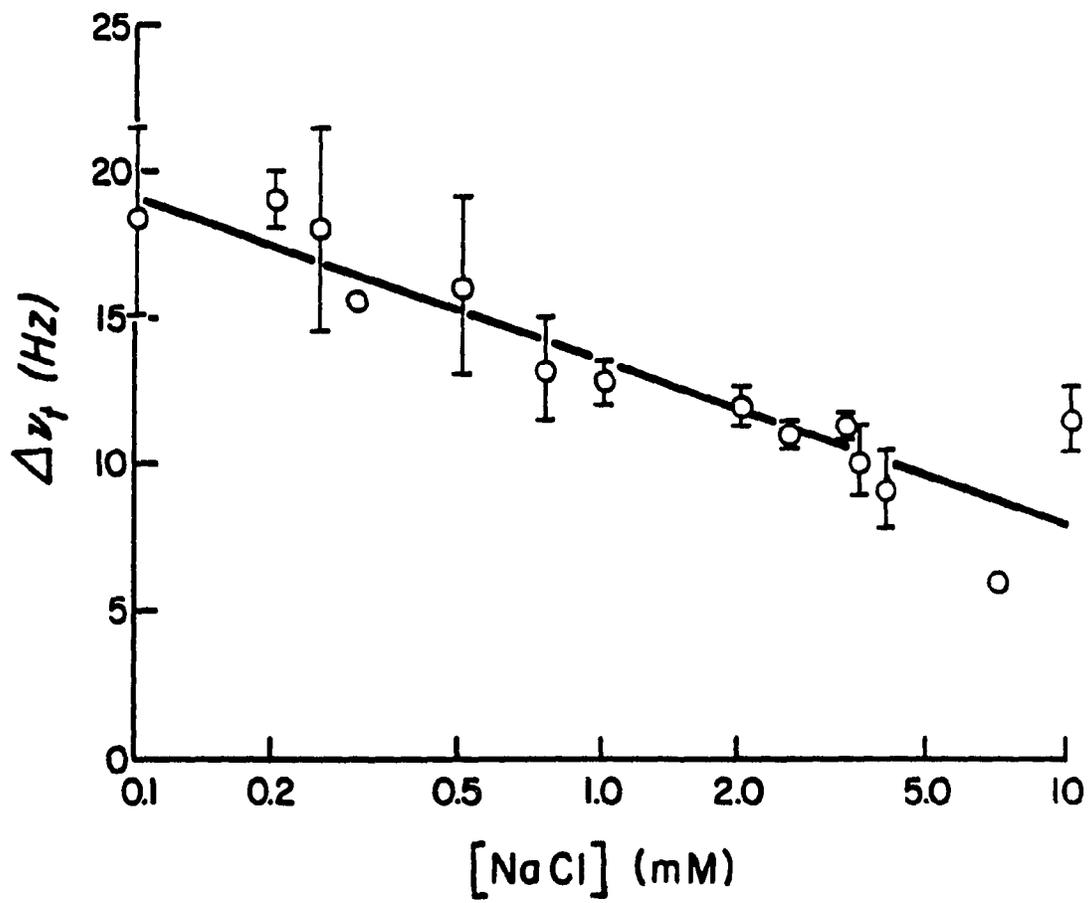


Figure 23.  $^{35}\text{Cl}$ -NMR binding curve for heated thylakoids. Samples at  $1.0 \text{ mg Chl ml}^{-1}$  were heated at  $38^\circ\text{C}$  for 3 min in the absence of  $\text{Cl}^-$ . For a discussion of the significance of  $\Delta\nu_t$ , see equations 1 and 2 in Chapter 2. For details of  $^{35}\text{Cl}$ -NMR assays, see Materials and Methods, Chapter 2. Error bars show the sample standard error for 2-7 measurements of  $\Delta\nu_t$  from different preparations.



the native system (compare with Fig. 12). As a result, the heated thylakoids behave more like BSA (Fig. 15) in terms of their  $\text{Cl}^-$  binding behavior, although the linewidths for the thylakoids are much broader. PS II membranes (Fig. 24) appear to be less sensitive to heat treatment than are thylakoids, although this difference may reflect the stabilizing influence of 400 mM sucrose in the PS II buffer (see, e.g., Higashi et al., 1975). Nevertheless, the  $^{35}\text{Cl}$ -NMR binding curve for PS II membranes after heat treatment at  $38^\circ\text{C}$  shows considerable flattening of the maxima and minima, particularly at 0.3 mM  $\text{Cl}^-$ . The differential effect of heating on this linewidth maximum indicates that the  $\text{Cl}^-$  binding domains which may give rise to these maxima denature as independent units.

We also observed the excess  $^{35}\text{Cl}$ -NMR linewidth at 2.0 mM  $\text{Cl}^-$  in depleted thylakoids heated at various temperatures. Fig. 25 shows that the temperature dependence is quite complex, probably because of the creation of multiple unfolded states of the proteins in the membrane. The peak at  $25\text{--}32^\circ\text{C}$  may reflect the exposure and subsequent denaturation of sequestered  $\text{Cl}^-$  binding sites, since the appearance of the peak corresponds to the temperature range at which  $\text{Cl}^-$  depletion sensitizes the OEC to heat treatment. Measurement of thylakoids by differential scanning calorimetry has shown that there is an irreversible endothermic transition at  $44^\circ\text{C}$  which is associated with the loss of  $\text{O}_2$ -evolution (Cramer et al., 1981).

#### **D. Discussion**

The results of these heating experiments indicate that  $\text{Cl}^-$  binding plays an important role in stabilizing the structure of the OEC. As shown by the data in Figs. 19, 20, 23 and 24, the  $\text{Cl}^-$  binding sites

Figure 24.  $^{35}\text{Cl}$ -NMR binding curve for heated PS II membranes. Samples at  $0.5 \text{ mg Chl ml}^{-1}$  were heated at  $38^\circ\text{C}$  for 3 min in the absence of  $\text{Cl}^-$ . For details of  $^{35}\text{Cl}$ -NMR assays, see Materials and Methods, Chapter 2. Error bars show the sample standard error for 2-4 measurements of  $\Delta\lambda_t$  from 8 different preparations. The mean activity of the preparations was  $313 \mu\text{mol O}_2 (\text{mg Chl})^{-1} \text{ hr}^{-1}$ .

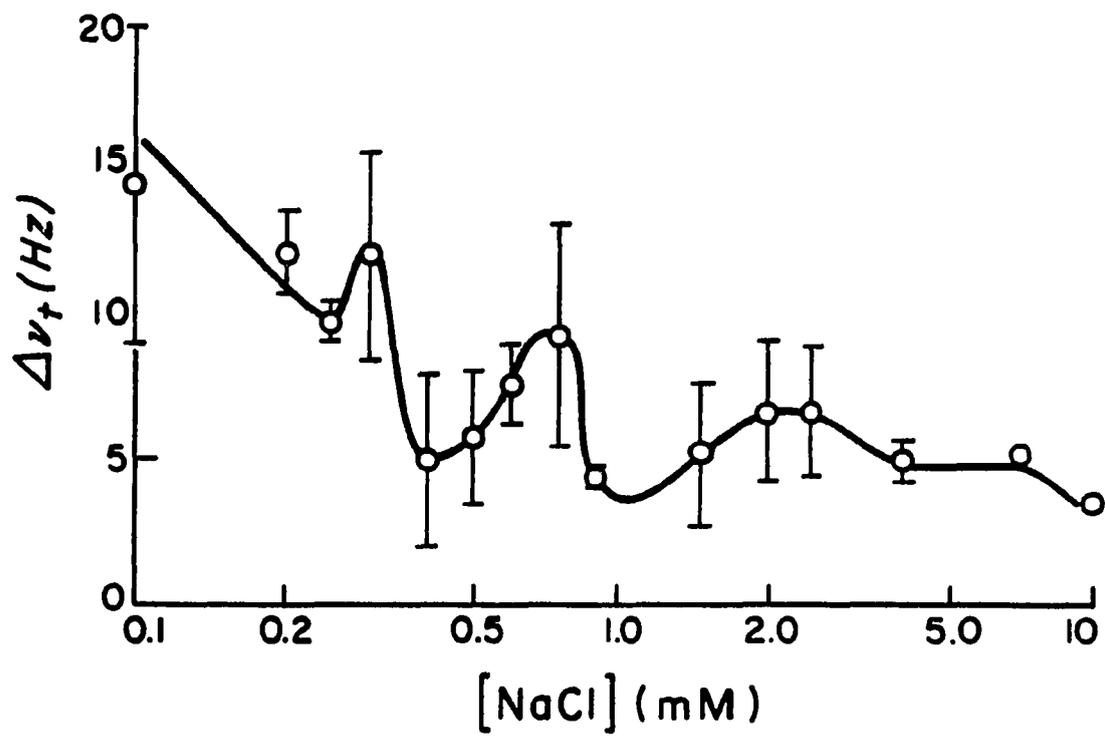
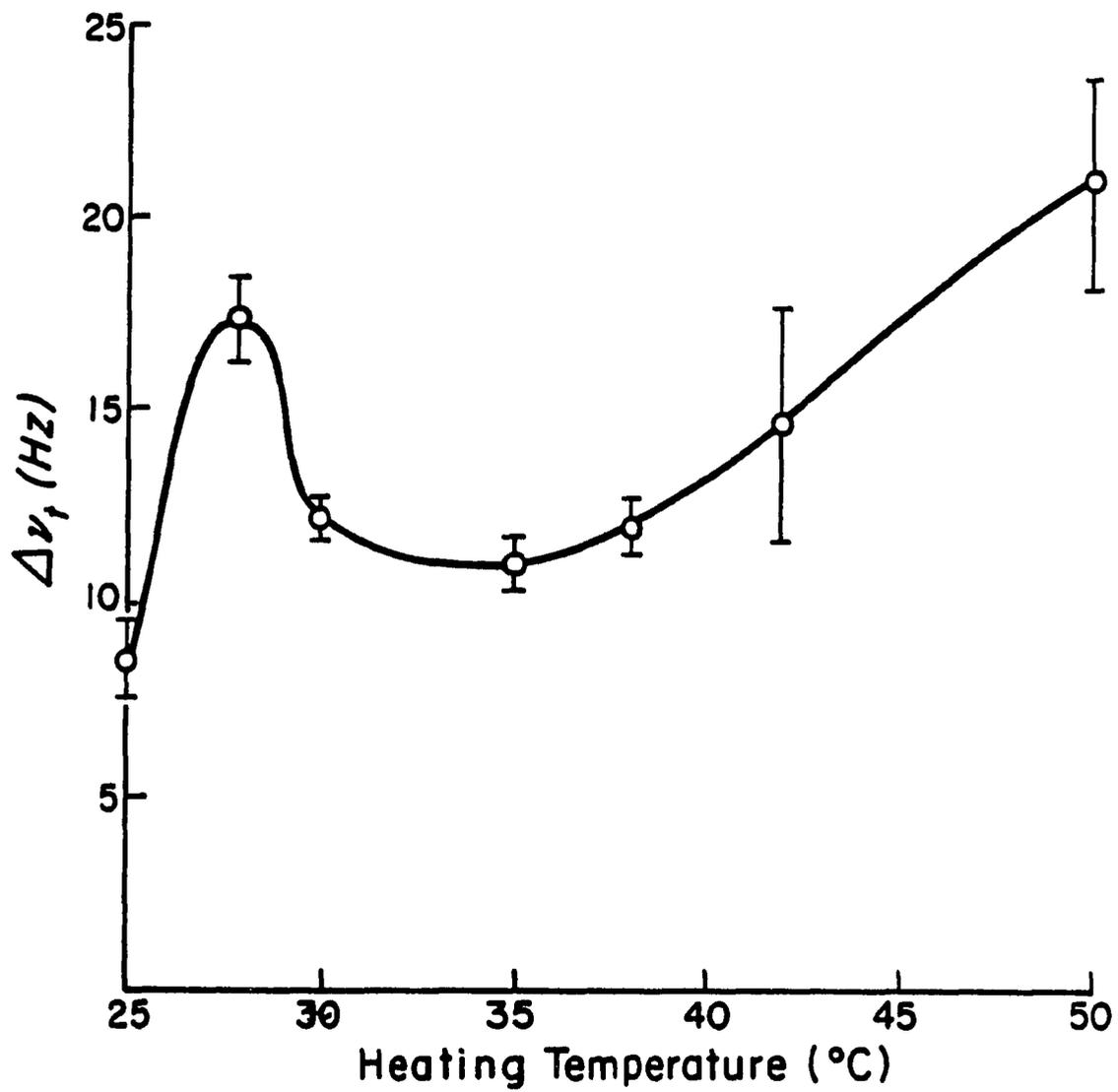


Figure 25. Effect of heating temperature on  $\Delta\mathcal{V}_t$  for thylakoids assayed in 2.0 mM  $\text{Cl}^-$ . Samples were heated at 1.0 mg Chl  $\text{ml}^{-1}$  for 3 min. For details of  $^{35}\text{Cl}$ -NMR assays, see Materials and Methods, Chapter 2. Error bars show the sample standard error for 2-5 measurements of  $\Delta\mathcal{V}_t$  from 8 different preparations.



themselves appear to be directly affected by heat treatment. The experiments in Fig. 18 suggest that their sensitivity to heating is more homogeneous when  $\text{Cl}^-$  is present, which creates a fairly sharp, cooperative transition from the active to the inactive state (Hinz, 1986). Studies on a number of proteins have demonstrated that ligand binding often stabilizes the protein structure against thermal denaturation (see, e.g., Webster and Gross, 1965; Zyk et al., 1969; Lee et al., 1973; Fukada et al., 1983). Fukada et al. have found, for example, that the enthalpy of unfolding measured for the L-arabinose binding protein from E. coli increases in the presence of arabinose, and that this increase is due to the additional enthalpy of dissociation of the ligand. This effect may have a parallel in the OEC, since Hind et al. (1969) have noted that increasing the temperature enhances the apparent dissociation of  $\text{Cl}^-$  from the thylakoid membrane. If  $\text{Cl}^-$  must first dissociate from the OEC before unfolding can occur, then this might explain why  $\text{Cl}^-$  raises the  $T_m$  for inactivation of the Hill reaction.

A previous study has indicated that the level of protection of the Hill activity is anion-specific, and that  $\text{Cl}^-$  is the most effective anion (Coleman et al., 1984). The effectiveness of other anions ( $\text{Br}^-$ ,  $\text{NO}_3^-$ , and  $\text{SO}_4^{2-}$ ) correlates with their ability to stimulate  $\text{O}_2$ -evolution in unheated thylakoids.

The loss of kinetic cooperativity following mild heat treatment, if it is analogous to the classical "desensitization" of allosteric enzymes (Gerhart and Pardee, 1962, 1963; Stadtman, 1966, 1970), suggests that  $\text{Cl}^-$  binding may involve both an active site and allosteric sites. There is a difficulty in interpreting such experiments, however, if the cooperativity involves subunit-subunit interactions. This is because

the loss of cooperativity following a particular treatment may simply result from dissociation of the enzyme subunits (Gerhart and Pardee, 1963), rather than from specific modification of the allosteric binding site(s). In PS II membranes, there is evidence that treatment at moderately high temperatures ( $>40^{\circ}\text{C}$ ) releases some of the extrinsic polypeptides from the OEC (Nash et al., 1985).

It is clear from Figs. 21 and 22 that any role for  $\text{Cl}^-$  at the OEC active site probably does not involve direct ligation or stabilization of Mn, since the stability of the Hill reaction shows a definite dependence on  $\text{Cl}^-$  in the temperature range ( $25^{\circ}$ - $35^{\circ}\text{C}$ ) at which no Mn release is observed. This finding is consistent with the results of EXAFS studies by Yachandra et al. (1986), who showed that  $\text{Cl}^-$  is not coordinated to Mn in the  $\text{S}_1$  state. We observed, moreover, that Mn release at higher temperatures is slightly greater when  $\text{Cl}^-$  is present. Earlier reports of a direct correlation between heat release of Mn and loss of  $\text{O}_2$ -evolution (Kimimura and Katoh, 1972; Cramer et al, 1981) may have been affected by the presence of very loosely-bound, non-functional Mn (Wydrzynski, 1982), which was substantially removed by EDTA treatment in our preparations. Using PS II particles, Nash et al. (1985) have reported the correlation between loss of  $\text{O}_2$ -evolution and loss of Mn at high temperature that we also have observed, but they apparently did not examine this phenomenon at the lower temperatures. In addition, their data indicate that the level of Mn release at elevated temperatures is slightly greater in the absence of  $\text{Cl}^-$ .

## E. Summary

Our  $^{35}\text{Cl}$ -NMR results enable us to conclude that although Mn release may contribute to the process of thermal inactivation, the primary reason for the heat-induced loss of Hill activity and the loss of apparent cooperativity in  $\text{Cl}^-$  binding is the destruction of the  $\text{Cl}^-$  binding domains. Moreover, as indicated by the data in Fig. 24, it is possible that these domains denature independently (see also Chapter 2, Fig. 14). This kind of behavior has been observed in other proteins, such as papain (Tiktopulo and Privalov, 1978). The data presented here support the idea that the  $\text{Cl}^-$  activation mechanism requires a highly-ordered protein structure within the OEC.

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#### IV. THE LOCATION OF THE CHLORIDE BINDING SITES IN THE OXYGEN-EVOLVING COMPLEX OF PHOTOSYSTEM II

##### A. Introduction

One of the primary objectives of research into the mechanism of  $\text{Cl}^-$  activation of  $\text{O}_2$ -evolution has been to find the location of the  $\text{Cl}^-$  binding sites. The pioneering work of Izawa et al. (1969) and Kelley and Izawa (1978) with  $\text{Cl}^-$  depleted thylakoids narrowed the search for the functional site to the donor side of Photosystem II (PS II). More recently, with the development of procedures to isolate PS II-enriched membrane sheets, it has become possible to dissect the structure of the oxygen-evolving complex (OEC) by selectively removing extrinsic polypeptides that are electrostatically bound to the inner thylakoid membrane in the vicinity of the OEC (for reviews, see Ghanotakis and Yocum, 1985; Govindjee et al., 1985; Murata and Miyao, 1987). This reductive approach has provided a considerable amount of information about the effect of polypeptide removal on the kinetics of  $\text{Cl}^-$  activation.

Studies have shown that complete removal of the three extrinsic polypeptides ( $M_r$  18, 24 and 33 kD) lowers the maximum attainable activity to about 10-20% of the control rate and raises the  $\text{Cl}^-$  requirement several fold (to about 100-200 mM; Ono and Inoue, 1984; Miyao and Murata, 1984, 1985; Kuwabara et al., 1985). Supplementation of the assay medium with 5-10 mM  $\text{CaCl}_2$  raises the activity to about 40% of the control rate (Ono and Inoue, 1984; Miyao and Murata, 1985; Kuwabara et al., 1985). When only the 33 kDa extrinsic polypeptide is present, the system requires 20-30 mM NaCl to reach 26%-40% of the original activity (Nakatani, 1984; Ghanotakis et al., 1985; Miyao and

Murata, 1985), but this level can be increased to 80% by substituting 10 mM  $\text{CaCl}_2$  (Ghanotakis et al., 1985). The native complex has a  $\text{Cl}^-$  requirement of about 10 mM (Miyao and Murata, 1985). When the 18 kD and 24 kD polypeptides are removed by salt-washing, dialyzed against EGTA to remove  $\text{Ca}^{2+}$ , and added back to the depleted membranes, the observed  $\text{Ca}^{2+}$  requirement for maximum activity is about 500-700  $\mu\text{M}$  (Ghanotakis et al., 1984).

These experiments demonstrate that although progressive depletion of extrinsic polypeptides consistently lowers the apparent  $\text{Cl}^-$  and  $\text{Ca}^{2+}$  affinity of the OEC, complete removal of polypeptides does not eliminate the stimulatory effect of these ions. Harsher treatment, such as incubation in 0.8 M Tris, not only removes the extrinsic polypeptides, but also disrupts the catalytic Mn site, thereby eliminating  $\text{O}_2$ -evolution (Yamamoto et al., 1981; Murata et al., 1983).

It is possible to infer from these results that  $\text{Cl}^-$  and  $\text{Ca}^{2+}$  function over an extended region of the OEC, including sites that are close to the Mn active site. Following the trail of  $\text{Cl}^-$  and  $\text{Ca}^{2+}$  by monitoring only  $\text{O}_2$ -evolution does not, however, provide enough specific information about the interaction of these ions with each of the polypeptides. The  $^{35}\text{Cl}$ -NMR results described in Chapters 2 and 3 indicate that  $\text{Cl}^-$  probably binds to multiple classes of sites comprised of highly-structured protein domains. The experiments in this chapter were designed to locate these domains with respect to the OEC polypeptides and the Mn active site. To do this, we examined the  $\text{Cl}^-$  binding properties of PS II membranes exposed to salt-washing (to remove some or all of the extrinsic polypeptides), Tris-washing (to remove the extrinsic polypeptides and the catalytic Mn), and hydroxylamine

treatment (to remove the Mn). Murata et al. (1983) have summarized the effects of these treatments on  $O_2$ -evolution and polypeptide/Mn depletion. By directly monitoring the effects of these treatments on  $Cl^-$  binding, we have been able to construct a more detailed map of the  $Cl^-$  binding sites within the OEC.

## B. Materials and Methods

PS II membranes were prepared as described in Chapter 2. Following the first centrifugation after Triton treatment, the pellet was rinsed with  $Cl^-$  free buffer containing 400 mM sucrose and 20 mM MES (pH 6.0). The upper pellet was resuspended in a small volume of  $Cl^-$  free buffer and then diluted into a larger volume of the same buffer containing a final concentration of 1.0 M NaCl, 1.0 M  $CaCl_2$ , or 1.5 mM  $NH_2OH$ -sulfate. For Tris treatment, the buffer employed was 0.8 M Tris-phosphate at pH 8.0. The final Chl concentration was approximately  $200 \mu g \text{ ml}^{-1}$ . The suspension was placed on ice and stirred in darkness for 25 min. For reconstitution experiments, the suspension that had been incubated in 1.0 M NaCl was diluted with  $Cl^-$  free buffer to a final NaCl concentration of 50 mM. This suspension was then allowed to stir on ice for an additional 25 min.

After treatment, the samples were transferred to centrifuge tubes and centrifuged at  $60,000 \times g$  for 40 min. The pellet was resuspended in a large volume of  $Cl^-$  free buffer and centrifuged for 20 min at  $40,000 \times g$ . This washing cycle was repeated two more times to remove loosely-associated  $Cl^-$ . The preparation was then given a final pH-jump treatment to remove tightly-bound  $Cl^-$  (see Materials and Methods, Chapter 2).

Thoroughly  $Cl^-$  depleted thylakoids, prepared as described in

Chapter 2, were given a 25 min treatment in 0.8 M Tris-phosphate (pH 8.0) and then washed twice in  $\text{Cl}^-$  free buffer (50 mM HEPES, pH 7.2). They were then resuspended in the same buffer. Details for activity and  $^{35}\text{Cl}$ -NMR assays are given in Chapter 2 and in the figure legends.

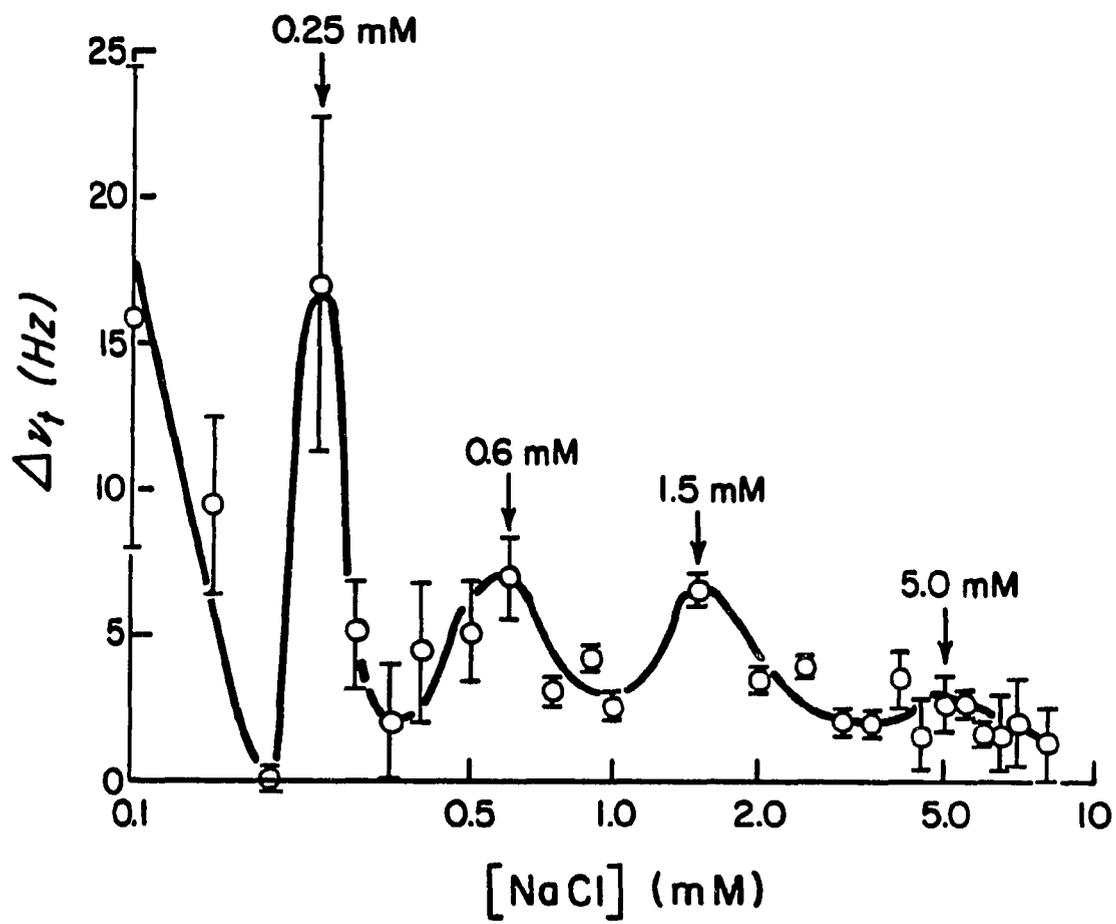
### C. Results and Discussion

Fig. 26 shows the effect of treatment with 1.5 mM  $\text{NH}_2\text{OH}$  on the  $\text{Cl}^-$  binding properties of PS II membranes. It is clear from comparison with untreated PS II membranes (Fig. 13, Chapter 2; and Fig. 28 A) that  $\text{NH}_2\text{OH}$  pre-treatment does not alter the overall shape of the  $^{35}\text{Cl}$ -NMR binding curve. Murata et al. (1983) have shown that while treatment with 1.5 mM  $\text{NH}_2\text{OH}$  removes more than half of the PS II Mn, it depletes the membranes of only 15% of the 18 kD and 24 kD polypeptides and does not affect the 33 kD polypeptide. The only observable effect on  $\text{Cl}^-$  binding of this selective depletion of Mn is a small shift in the  $[\text{Cl}^-]$  for each of the  $^{35}\text{Cl}$ -NMR linewidth maxima: from 0.3 to 0.25 mM (17%), from 0.75 to 0.6 mM (20%), from 2.0 to 1.5 mM (25%), and from 7.0 to  $\sim$ 5.0 mM (29%). The latter peak falls within the noise level.

The reason for the shift in the  $\text{Cl}^-$  concentration dependence of the binding curve is not known. It is possible that Mn depletion relieves constraints on the motion of the polypeptide chains, and consequently makes them more sensitive to changes in  $[\text{Cl}^-]$ . In any case, the observation that removal of Mn does not substantially affect the  $\text{Cl}^-$  binding properties of the OEC (as observed by  $^{35}\text{Cl}$ -NMR) indicates that the Mn itself is not a binding site for  $\text{Cl}^-$  in dark-adapted PS II membranes. This finding is consistent with the heating results discussed in Chapter 3.

Removal of the 18 kD and 24 kD polypeptides by washing the PS II

Figure 26.  $^{35}\text{Cl}$ -NMR binding curve for PS II membranes treated with 1.5 mM  $\text{NH}_2\text{OH}$ . The preparations showed no detectable  $\text{O}_2$ -evolution after  $\text{NH}_2\text{OH}$  treatment. Error bars show the sample standard error for 2-3 measurements on 4 different preparations. See Chapter 2 for NMR details.



membranes with 1.0 M NaCl (Miyao and Murata, 1983) raises the apparent activator dissociation constant for  $\text{Ca}^{2+}$  to about 6.9 mM in the presence of 50 mM  $\text{Cl}^-$  (Fig. 27). The maximum attainable activity under these conditions is calculated to be  $221 \mu\text{mol O}_2 (\text{mg Chl})^{-1} \text{hr}^{-1}$ , or about 60% of the control rate. The level of reactivation is consistent with reports from other laboratories (e.g., Ghanotakis et al., 1985), and indicates an apparent OEC requirement for  $\text{Ca}^{2+}$  that is beyond typical physiological concentrations.

The effect of NaCl washing on the  $^{35}\text{Cl}$ -NMR binding curve for PS II membranes is striking. Fig. 28 B shows that this treatment causes two major changes in the binding curve: 1) it substantially reduces the line-broadening at very low  $[\text{Cl}^-]$  ( $\sim 0.1$  mM) and at high  $[\text{Cl}^-]$  (1.0-10 mM), indicating that the enzyme affinity for  $\text{Cl}^-$  is also reduced; and 2) it replaces the linewidth maxima at 0.3 mM and 0.75 mM  $\text{Cl}^-$  with linewidth minima, while simultaneously broadening the other linewidths at 0.2 mM and 0.4-0.5 mM  $\text{Cl}^-$ . The correspondence between the linewidth maxima in Fig. 28 A and the linewidth minima in Fig. 28 B begins to disappear at  $[\text{Cl}^-] > 1.0$  mM.

The persistence of sharp, concentration-dependent changes in  $\Delta\nu_t$  in NaCl-washed membranes suggests that  $\text{Cl}^-$ -dependent conformational changes can still occur in these samples. However, the transformation from increased affinity (linewidth maxima) to decreased affinity (linewidth minima) at 0.3 mM and 0.75 mM  $\text{Cl}^-$ , along with the overall loss of  $\text{O}_2$ -evolution (to approximately 7% of the control activity in 50 mM  $\text{Cl}^-$ ), suggests that whatever changes are triggered by adding  $\text{Cl}^-$  gradually generate enzyme conformations increasingly unfavorable to  $\text{Cl}^-$  binding.

Since it is known that the addition of high concentrations of  $\text{Ca}^{2+}$  may partially replace the function of the 18 kD and 24 kD polypeptides

Figure 27. Double-reciprocal plot for  $\text{Ca}^{2+}$  activation of PS II membranes washed with 1.0 M NaCl. Oxygen evolution was measured at pH 6.0 in the presence of 50 mM  $\text{Cl}^-$ .

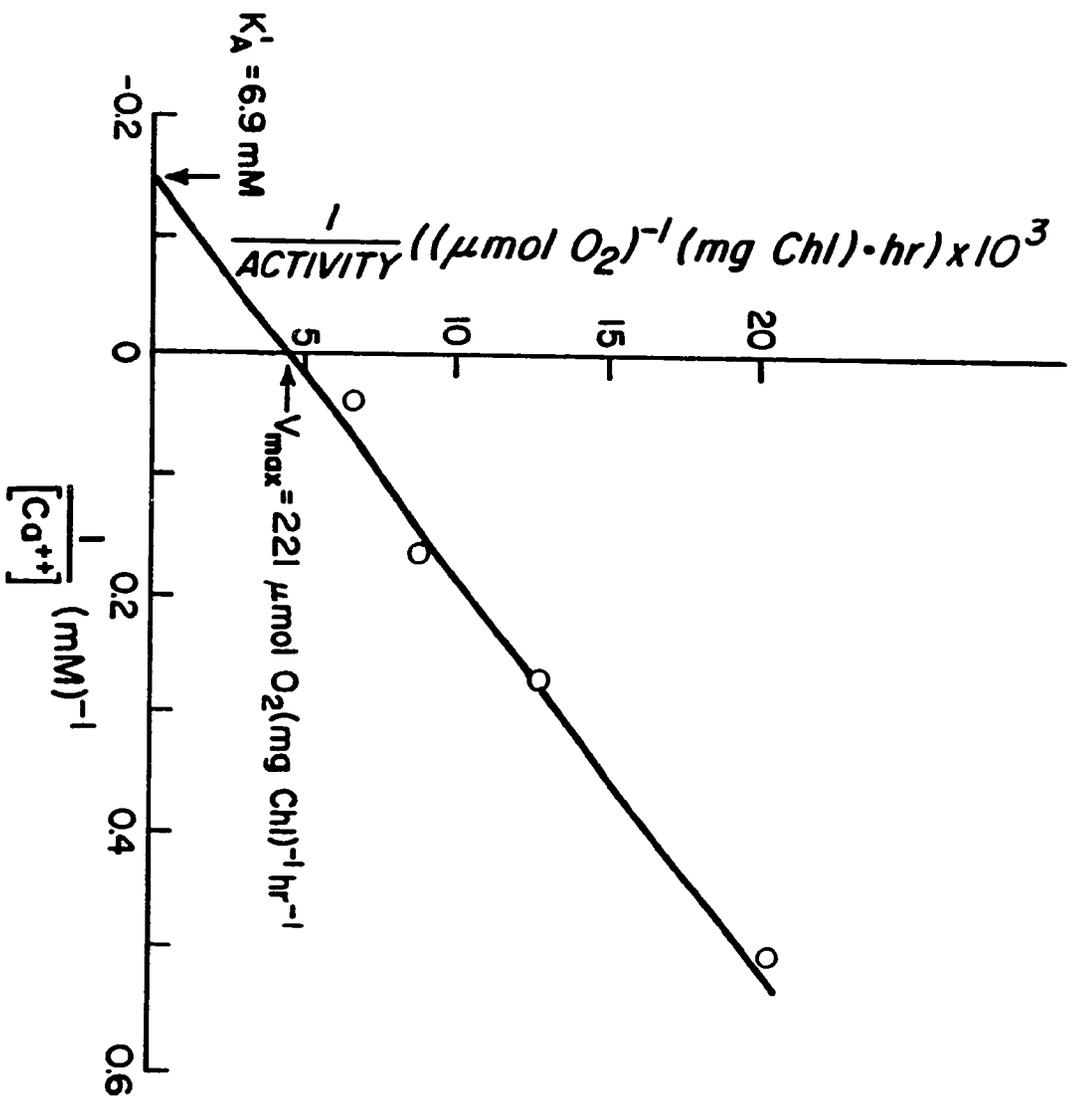
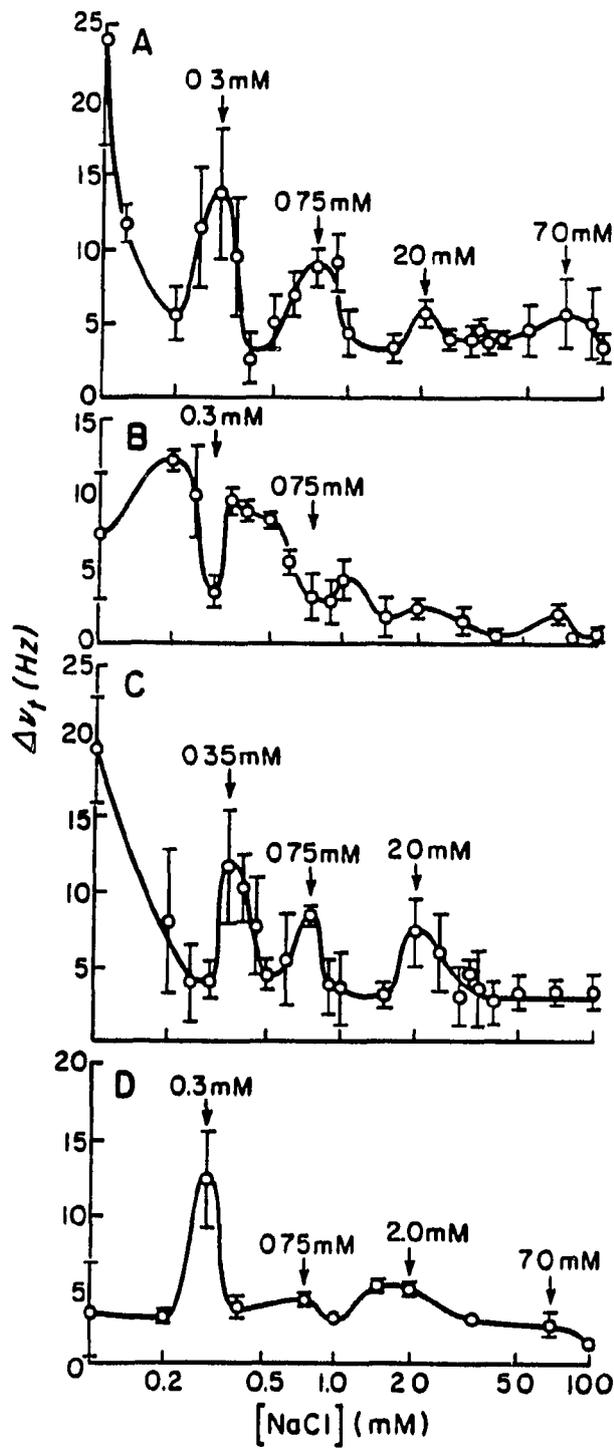


Figure 28. Effect of polypeptide depletion and reconstitution on the  $^{35}\text{Cl}$ -NMR binding curve for PS II membranes. (A) Untreated membranes (mean activity =  $383 \mu\text{mol O}_2 (\text{mg Chl})^{-1} \text{ hr}^{-1}$  in 50 mM  $\text{Cl}^-$ ). (B) Membranes washed in 1.0 M NaCl (mean activity =  $28 \mu\text{mol O}_2 (\text{mg Chl})^{-1} \text{ hr}^{-1}$  in 50 mM  $\text{Cl}^-$ ); error bars show the sample standard error for 2-4 measurements on 4 different preparations. (C) Membranes washed in 1.0 M NaCl and assayed in the presence of 2.0 mM  $\text{CaSO}_4$  (mean activity =  $102 \mu\text{mol O}_2 (\text{mg Chl})^{-1} \text{ hr}^{-1}$  in 50 mM NaCl + 2.0 mM  $\text{CaSO}_4$ ); error bars show the sample standard error for 2-5 measurements on 6 different preparations. (D) Membranes washed in 1.0 M NaCl and reconstituted by diluting the  $[\text{Cl}^-]$  to 50 mM (mean activity =  $122 \mu\text{mol O}_2 (\text{mg Chl})^{-1} \text{ hr}^{-1}$  in 50 mM NaCl); error bars show the sample standard error for 2-3 measurements on 3 preparations.



in stimulating  $O_2$ -evolution in these preparations, we added a fixed concentration of  $Ca^{2+}$  (in the form of 2.0 mM  $CaSO_4$ ) to the  $^{35}Cl$ -NMR assay buffer and measured the  $^{35}Cl$ -NMR binding curve again. Fig. 28 C shows that the addition of 2.0 mM  $Ca^{2+}$  almost completely restores three of the four  $^{35}Cl$ -NMR linewidth maxima that were observed in the control (Fig. 28 A). Restoration of the linewidth maximum at 7.0 mM was not observed, however, and the linewidth maximum at 0.3 mM  $Cl^-$  appeared to be shifted slightly upward in concentration (to 0.35 mM  $Cl^-$ ).

Our  $^{35}Cl$ -NMR results with  $Ca^{2+}$  parallel the findings of a number of other studies on  $O_2$ -evolution, in which  $Ca^{2+}$  was found to partially replace the function of the 18 kD and 24 kD polypeptides. The reappearance of the  $^{35}Cl$ -NMR linewidth maxima after  $Ca^{2+}$  addition indicates that this cation reorganizes the complex into a quasi-native structure that is then able to bind  $Cl^-$  and evolve oxygen. As a result, the conformational changes giving rise to the  $^{35}Cl$ -NMR linewidth maxima appear to occur in a more orderly sequence as the  $[Cl^-]$  increases. This experiment also indicates that the 18 kD and 24 kD polypeptides are not directly involved in  $Cl^-$  binding to the sequestered sites.

The effects of NaCl-washing on the  $^{35}Cl$ -NMR binding curve are partially reversible. The data in Fig. 28 D demonstrate that dilution of the PS II suspension from 1.0 M to 50 mM NaCl during preparation enables the functional complex to re-assemble, although the restoration is incomplete. The linewidth maxima at 0.3 mM and 2.0 mM  $Cl^-$  appear to be restored more completely (with  $\Delta\nu_t$  measured at 90% and 86% of the control value, respectively) than are the maxima at 0.75 mM and 7.0 mM  $Cl^-$  (with  $\Delta\nu_t$  measured at 49% and 47% of the control value, respectively). The line-broadening at 0.1 mM  $Cl^-$  is, however, substantially reduced. The observation of partial reconstitution of the

$^{35}\text{Cl}$ -NMR linewidth maxima is consistent with the partial reconstitution of  $\text{O}_2$ -evolution in these preparations (approximately 30% of the control rate in 50 mM  $\text{Cl}^-$  with no added  $\text{Ca}^{2+}$ ). These  $^{35}\text{Cl}$ -NMR results support the findings of Miyao and Murata (1986), who reported that dilution of NaCl-treated PS II membranes to 34 mM NaCl partially restored the control rate of  $\text{O}_2$ -evolution and enabled the 18 kD and 24kD polypeptides to re-bind to the membrane.

The ability of  $\text{Ca}^{2+}$  (at high concentrations) to mimic precisely the function of the missing 18 kD and 24 kD polypeptides (as reflected in the restoration of both  $\text{Cl}^-$  binding and enzyme activity) suggests that it may function by coordinating to a number of negatively charged residues on the remaining proteins. It is possible that these negative charges are normally associated with positively charged residues on the 18kD and/or 24 kD polypeptide(s) in the native complex.  $\text{Ca}^{2+}$  might then replace these two polypeptides, but with reduced effectiveness. It is also possible that these two polypeptides raise the  $\text{Ca}^{2+}$  affinity of the complex directly, by supplying ligands to a specific  $\text{Ca}^{2+}$  binding site, or indirectly, by increasing the affinity of other proteins for  $\text{Ca}^{2+}$ . As far as  $\text{Cl}^-$  binding is concerned, it is apparent from these data that the  $\text{Cl}^-$  binding sites revealed in the  $^{35}\text{Cl}$ -NMR linewidth maxima do not reside on the 18 kD and 24kD polypeptides, although these two polypeptides play an important role in stabilizing their structure.

To determine whether the presence of the 33 kD extrinsic polypeptide is required in order to observe the  $^{35}\text{Cl}$ -NMR linewidth maxima, we treated PS II membranes with 1.0 M  $\text{CaCl}_2$ . Ono and Inoue (1983) have shown that this treatment removes all of the extrinsic polypeptides. The  $^{35}\text{Cl}$ -NMR binding curve for this preparation (Fig. 29) exhibits a single, broad linewidth maximum centered at about 0.5 mM  $\text{Cl}^-$ .

No other maxima were observed in the  $\text{Cl}^-$  concentration range between 0.1 and 10 mM, and supplementation of the medium with 2.0 mM  $\text{CaSO}_4$  had no additional effect. We conclude from this result that a significant amount of high-affinity  $\text{Cl}^-$  binding is still possible after depletion of the extrinsic polypeptides. If this binding involves portions of the intrinsic D1/D2 proteins (see Fig. 1 for a current picture of PS II structure), then it may conceivably influence the water-splitting reactions at the Mn active site. In any case, it is clear from a comparison of this  $^{35}\text{Cl}$ -NMR binding curve with that of the intact system (Fig. 28 A) or the preparation washed with NaCl and supplemented with  $\text{Ca}^{2+}$  (Fig. 28 C), that the 33 kD polypeptide must be present in order to observe the multiple linewidth maxima characteristic of the native complex.

When thylakoids or PS II membranes are treated with 0.8 M Tris, which inactivates  $\text{O}_2$ -evolution and releases the catalytic Mn (Yamamoto et al., 1981; Murata et al., 1983), most of the specific  $\text{Cl}^-$  binding is eliminated (Figures 30 and 31). This result is a further indication that the  $\text{Cl}^-$  binding observed in the  $\text{CaCl}_2$ -washed PS II membranes is associated with proteins involved in Mn binding.

#### D. Summary

We can briefly summarize the significance of the results presented in this chapter by concluding: 1) Under dark-adapted conditions,  $\text{Cl}^-$  does not bind directly to the Mn in the OEC; 2) the  $\text{Cl}^-$  binding mechanism in the intact complex involves the cooperation of both intrinsic sites (possibly on D1/D2) and extrinsic sites (on the 33 kD polypeptide); and 3)  $\text{Cl}^-$  binding to the extrinsic sites is enhanced by the indirect cooperation of the 18 kD and/or 24 kD polypeptides. The

Figure 29.  $^{35}\text{Cl}$ -NMR binding curve for PS II membranes washed with 1.0 M  $\text{CaCl}_2$ . Activity was approximately  $46 \mu\text{mol O}_2 (\text{mg Chl})^{-1} \text{hr}^{-1}$  in the presence of 25 mM  $\text{CaCl}_2$ .

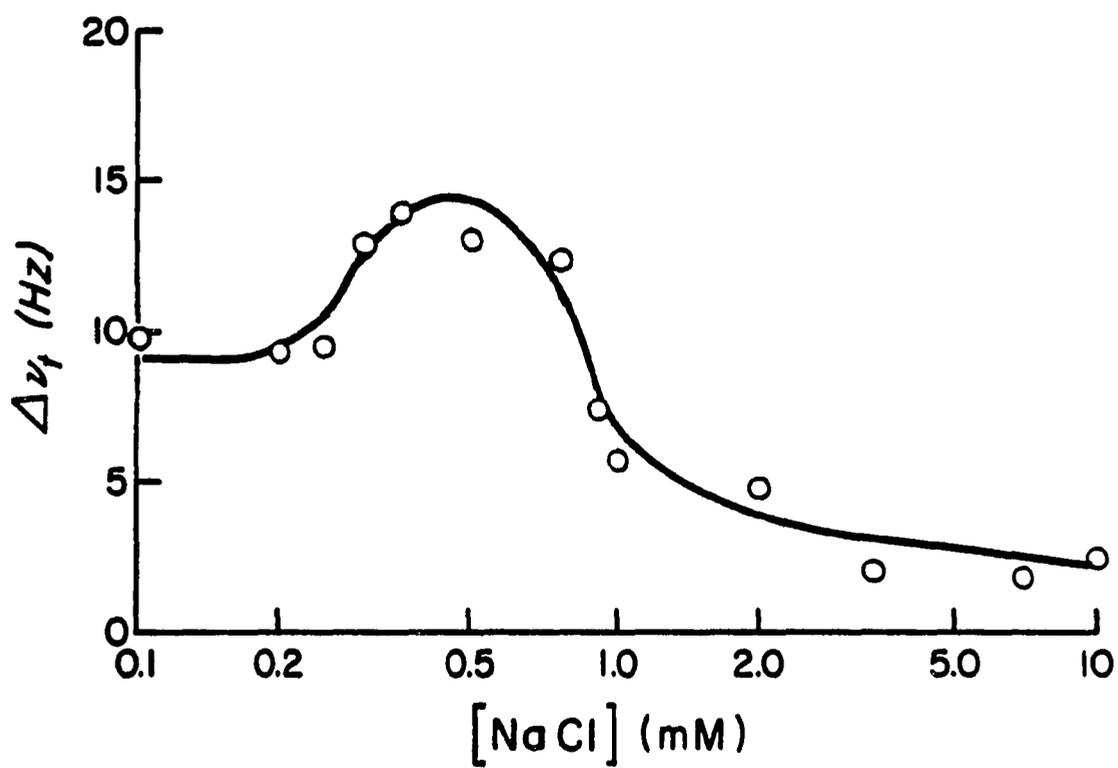


Figure 30.  $^{35}\text{Cl}$ -NMR binding curve for thylakoids washed with 0.8 M Tris. Thylakoids at a concentration of  $1.0 \text{ mg Chl ml}^{-1}$  were suspended in 50 mM HEPES at pH 7.2. Tris treatment eliminated an average of 67% of the Hill activity, measured in 50 mM  $\text{Cl}^-$ . Error bars show the variation in  $\Delta\nu_t$  for 2 measurements on 4 different preparations.

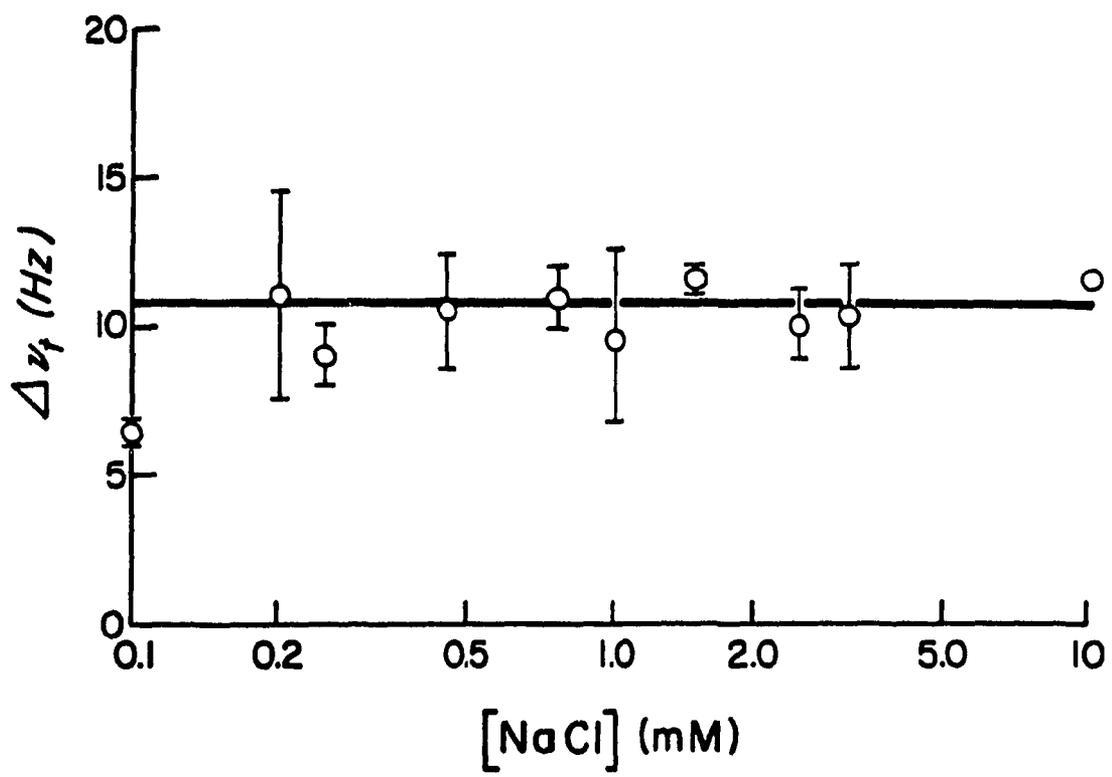
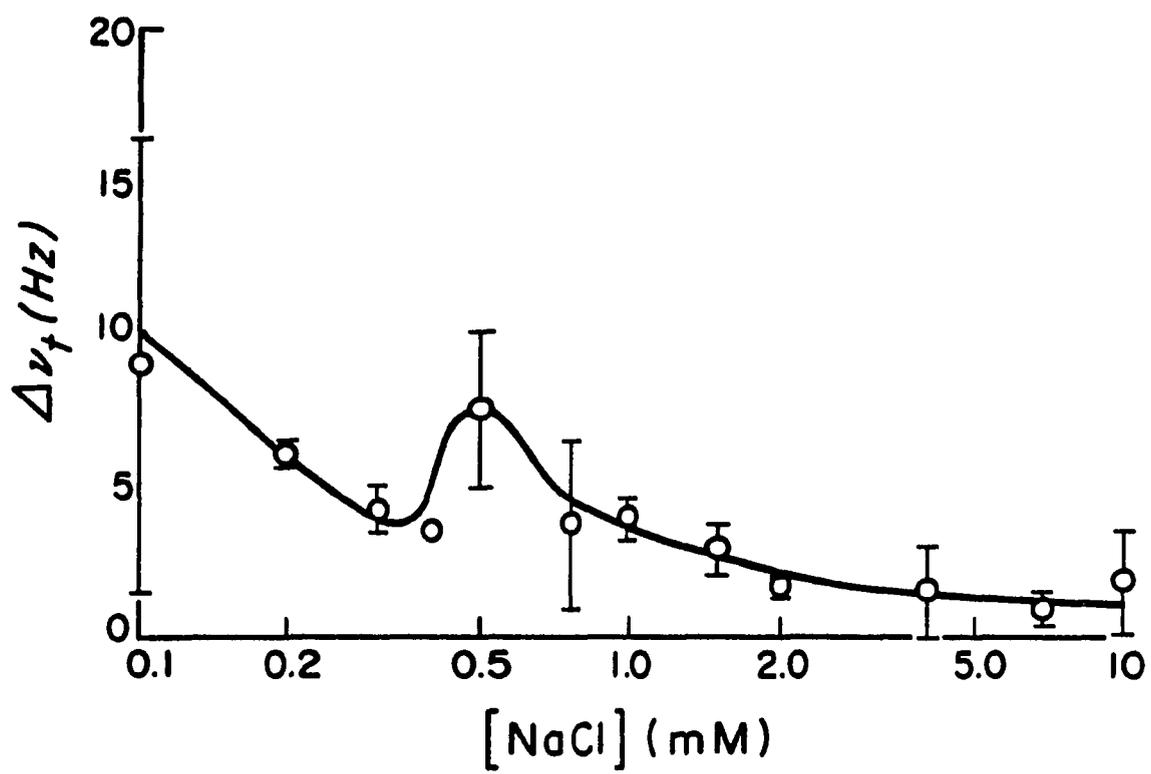


Figure 31.  $^{35}\text{Cl}$ -NMR binding curve for PS II membranes washed with 0.8 M Tris. The mean rate of  $\text{O}_2$ -evolution after Tris treatment was  $16 \mu\text{mol O}_2 (\text{mg Chl})^{-1} \text{ hr}^{-1}$  (approximately 96% inhibition). Error bars show the variation in  $\Delta\nu_t$  for 2 measurements on 2 different preparations.



function of these two polypeptides can be partially replaced by a high concentration of  $\text{Ca}^{2+}$  (2.0 mM). A hypothesis to explain the mechanism of  $\text{Cl}^-$  activation of the OEC is presented in Chapter 5.

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## V. A MODEL FOR THE MECHANISM OF CHLORIDE ACTIVATION OF OXYGEN EVOLUTION IN PHOTOSYSTEM II

### A. Introduction

Although  $\text{Cl}^-$  is a very simple anion, its influence on the function of the oxygen-evolving complex (OEC) of Photosystem II (PS II) is profound. The  $^{35}\text{Cl}$ -NMR experiments described in Chapters 2-4 of this thesis were undertaken in order to provide more direct insight into the number, location, and significance of the  $\text{Cl}^-$  binding sites in spinach PS II. The purpose of this chapter is to combine the information obtained from the  $^{35}\text{Cl}$ -NMR experiments with what is known from other sources into a simple, consistent, and practical model for the mechanism of  $\text{Cl}^-$  activation.

The approach that will be used here will be to: 1) define the essential features of the  $\text{Cl}^-$  binding sites as indicated by the experimental evidence; 2) compare these features with similar, more thoroughly characterized structures in other proteins or models; and 3) identify probable sites in PS II proteins whose structure and composition would be consistent with these criteria.

There are several lines of evidence indicating that the  $\text{Cl}^-$  binding sites in the OEC are not homogeneous. Homann (1985) has found, for example, that when the pH-dependence of  $\text{Cl}^-$  binding is measured by assaying its effect on the light-driven steady-state turnover of the enzyme,  $\text{Cl}^-$  appears to bind to a group having a  $\text{pK}_a$  of about 6.0. Studies of  $\text{Cl}^-$  depletion, however, have shown that the OEC retains most of its  $\text{Cl}^-$  unless the pH of the medium is raised beyond 8.0 (Izawa et al., 1969; Theg and Homann, 1982; Homann et al., 1983). Similarly, the  $^{35}\text{Cl}$ -NMR binding studies reported in Chapters 3 and 4 of this thesis

indicate that although the multiple linewidth maxima observed in measurements of the untreated membranes are eliminated by heating (Chapter 3, Fig. 23) or removal of the extrinsic polypeptides (Chapter 4, Fig. 28), a significant amount of  $\text{Cl}^-$  binding still occurs. Oxygen evolution is also not completely eliminated by these two treatments. A simple explanation for these apparent anomalies is that the OEC contains two different types of  $\text{Cl}^-$  binding sites, and that they are physically separate and chemically distinct. One site, located nearer to the Mn active site, will be referred to here as "intrinsic". The other site, located on the 33 kD polypeptide will be referred to as "extrinsic".

#### **B. Proposed Structure and Properties of the Intrinsic $\text{Cl}^-$ Binding Site**

One of the major effects of  $\text{Cl}^-$  on the function of the OEC active site is a pronounced shift of the activity-pH optimum to alkaline pH (Gorham and Clendenning, 1952; Critchley, 1985). Massey (1953) and Alberty (1954) have described in general terms how this phenomenon can be attributed to the presence of three protonatable groups in the enzyme active site (see Chapters 1 and 2). In applying this idea to the OEC, we make the assumption that the anion-sensitive, bell-shaped pH profile arises because one of these groups is a proton acceptor (unprotonated at the pH optimum), the second is a proton donor (protonated at the pH optimum), and the third is capable of binding  $\text{Cl}^-$  in its protonated state. Combination of  $\text{Cl}^-$  with this third group would be expected to raise the  $\text{pK}_a$ 's of the other two, producing an alkaline shift in the activity-pH curve (Coleman and Govindjee, 1985). If the catalytic function of the first two groups is to remove protons from water, then the upward shift in their  $\text{pK}_a$ 's will activate the enzyme by increasing

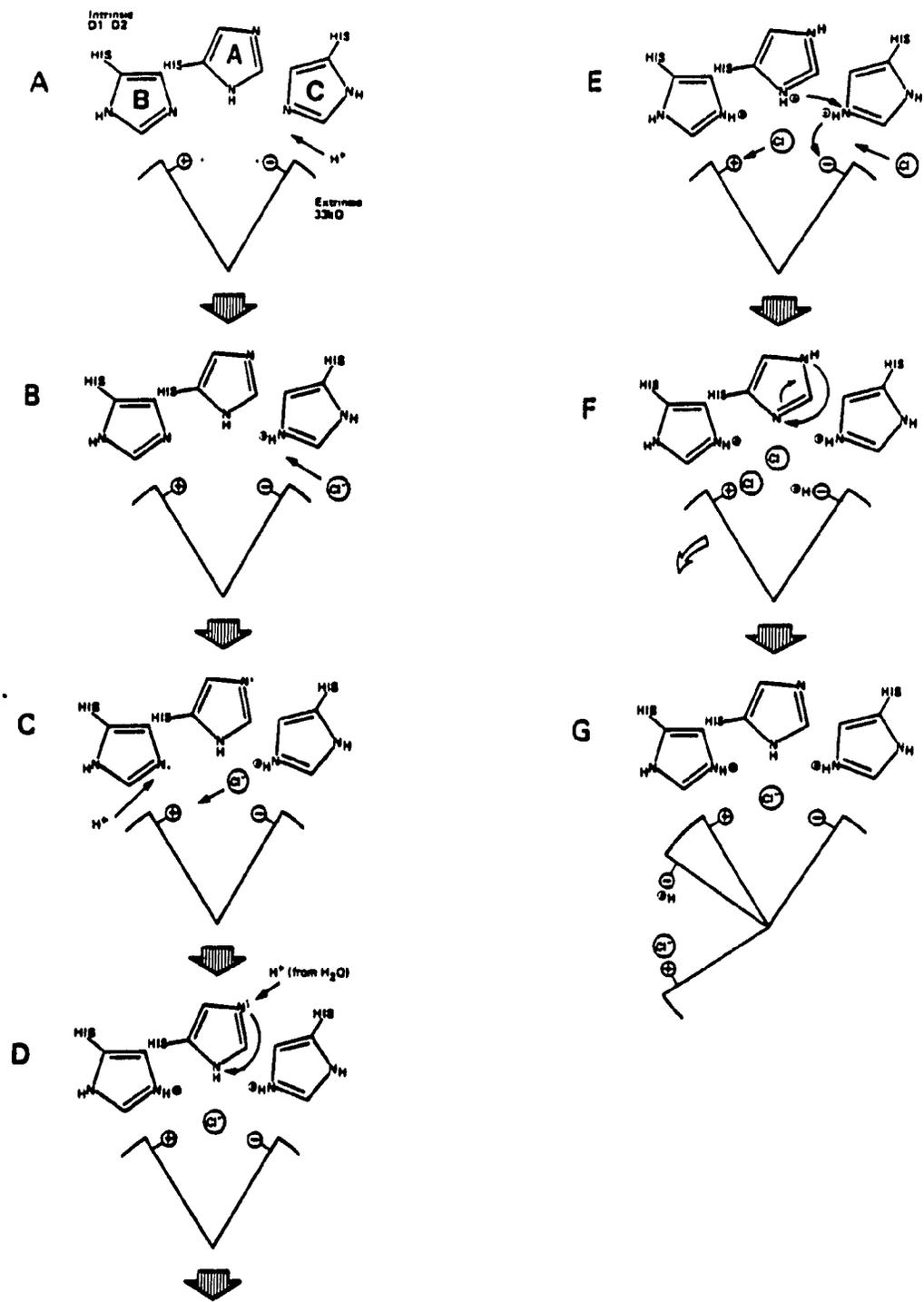
the rate of O<sub>2</sub>-evolution.

Homann's conclusion that the pK<sub>a</sub> of the Cl<sup>-</sup> binding group is at 6.0 places obvious constraints on the identity of the Cl<sup>-</sup> binding residue. In addition, the pH profile for Cl<sup>-</sup> binding, as measured by <sup>35</sup>Cl-NMR (Fig. 17, Chapter 2), indicates that there may be as many as three groups whose protonation states affect the binding of Cl<sup>-</sup> in the region between pH 5.4 and 6.5. Together, these data are consistent with the suggestion that three histidyl residues contribute to the active site mechanism of the OEC.

It is not uncommon for groups of histidines to be involved in the active sites of enzymes, particularly in those which catalyze acid-base reactions. In the active site of human carbonic anhydrase, for example, there are three histidines (with pK<sub>a</sub>'s of 4.72, 6.00, and 6.14) whose pK<sub>a</sub>'s are linked (Campbell et al., 1974; Forsman et al., 1983). In pancreatic ribonuclease, there are two catalytic histidines which constitute part of a Cl<sup>-</sup> binding site (Matthew and Richards, 1982). The protonation state of these two residues is strongly influenced by the presence of bound anions (Richards and Wyckoff, 1971 (see pp. 801-806); Matthew and Richards, 1982). Consequently, these anions directly influence the activity of the enzyme (Kalnitsky et al., 1959). The pK<sub>a</sub>'s of the histidines also depend on the nature of the anion itself (Richards and Wyckoff, 1971).

The mechanism we propose for this site (Fig. 32) involves facilitated transfer of protons (Wang, 1968) from bound water at the active site Mn to protonatable groups on the 33 kD extrinsic polypeptide. The transfer pathway involves three histidine imidazole groups whose pK<sub>a</sub>'s are raised by the presence of Cl<sup>-</sup>. Initially, Cl<sup>-</sup> binding to this intrinsic site in the Cl<sup>-</sup> depleted system accelerates

Figure 32. A model for the  $\text{Cl}^-$  binding mechanism at the active site of the OEC. As the pH of the external medium is lowered (A), His-C is protonated, enabling  $\text{Cl}^-$  to bind (B).  $\text{Cl}^-$  binding to His-C raises the  $\text{pK}_a$  of His-B, so that as the pH is lowered further to 6.0, His-B is protonated (C).  $\text{Cl}^-$  binding also raises the  $\text{pK}_a$  of His-A, which is able to accept a  $\text{H}^+$  from the water-splitting reactions (D). When the  $\text{H}^+$  is moved into the  $\text{Cl}^-$  binding site by a resonance shift in the imidazole group (see Metzler, 1977, p. 55) of His-A (E), the site is destabilized. Because of this instability,  $\text{Cl}^-$  and  $\text{H}^+$  attack a salt-bridge on the 33 kD polypeptide (E). His-A then relaxes, and another  $\text{Cl}^-$  binds (F). Another extrinsic site is then open to attack (G).

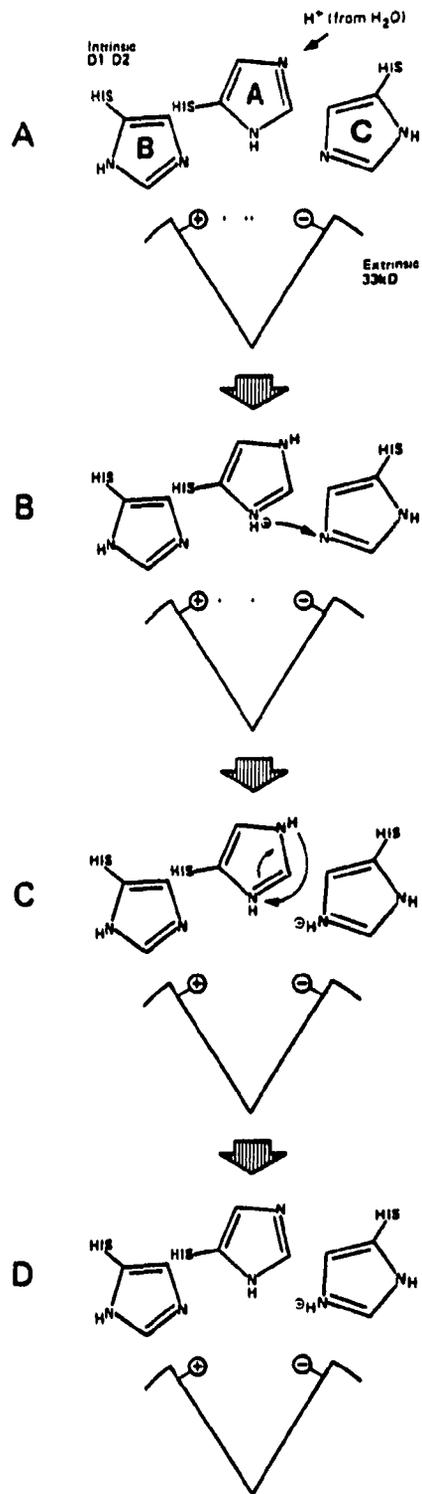


the light-driven dissociation of  $H^+$  from water by stabilizing the  $H^+$  on the histidine cluster. In subsequent steps, it connects the intrinsic site to the extrinsic sites by promoting the transfer of protons into previously sequestered (salt-bridged) domains of the 33 kD polypeptide. In the absence of  $Cl^-$  (Fig. 33), the intrinsic site is able to accept a single  $H^+$  from water (depending on the pH), but cannot transfer this  $H^+$  to the extrinsic sites. The details of the individual steps are given in Figures 32 and 33. The kinetics of  $H^+$  transfer between water and imidazole indicate that an imidazole group is an appropriate acceptor for  $H^+$  donated from water (Metzler, 1977 (see pp. 405-406)).

### C. Justification for the Mechanism at the Intrinsic Site

This model explains a number of experimental observations concerning the catalytic effects of  $Cl^-$  on the water-splitting reactions (Fig. 32): 1) The  $pK_a$  for optimal activation by  $Cl^-$  is 6.0 because at this pH,  $Cl^-$  is bound to a protonated His-C. His-B is also protonated at this pH, and therefore is able to donate a proton to an acceptor group on the extrinsic sites. His-A is unprotonated, and binds a  $H^+$  from water. 2) Chloride shifts the activity-pH optimum by neutralizing some of the positive charge in the cluster. By raising the  $pK_a$  of His-A, it also raises the maximum velocity of the enzyme at alkaline pH. 3) The activity of the enzyme depends on the nature of the anion because the magnitude of the  $pK_a$  shifts depends on the contribution of the anion to the electrostatic potential (Matthew and Richards, 1982; Mehler and Eichele, 1984). 4) In the absence of  $Cl^-$  (Fig. 33), the OEC cannot advance beyond the  $S_2$  state (Itoh, et al., 1984; Theg et al., 1984; see also Chapter 1) because the  $Cl^-$ -free site can only accept one  $H^+$ . This  $H^+$  is held stably and at a shorter distance from the Mn site because

Figure 33. A model for proton transfer into the intrinsic  $\text{Cl}^-$  binding site in the absence of  $\text{Cl}^-$ . With no  $\text{Cl}^-$  present, His-A accepts a  $\text{H}^+$  from water (A) and transfers it to His-C, which has a higher  $\text{pK}_a$  (B). His-A then relaxes back to its original state (C). The  $\text{H}^+$  remains bound to His-C because there is no  $\text{Cl}^-$  present to attack the salt-bridge on the 33 kD polypeptide (D). The system cannot accept additional protons.



there is no  $\text{Cl}^-$  available to move it out onto the 33 kD polypeptide.

5) Amines will compete with  $\text{Cl}^-$  (see Sandusky and Yocum, 1986) at the intrinsic site by forming hydrogen bonds with the imidazole nitrogens, thereby displacing  $\text{Cl}^-$ .

#### **D. Location of the Intrinsic Site within the OEC**

Given that three histidyl residues appear to be required for the  $\text{Cl}^-$  activation mechanism at the active site, we decided to examine the published amino acid sequences of the intrinsic PS II proteins (particularly D1 and D2), to determine whether such a cluster might exist. The proteins D1 and D2 were chosen for several reasons: 1) They have recently been implicated as the PS II reaction center core polypeptides (Nanba and Satoh, 1987). 2) Both proteins are believed to have long loops of charged residues (including histidine) that project into the thylakoid lumen (Trebst and Draber, 1986). 3) The D2 protein is believed to contain binding sites for the catalytic Mn (Metz and Bishop, 1980). 4)  $^{35}\text{Cl}$ -NMR results show that PS II membranes in which the extrinsic polypeptides have been removed are still able to bind  $\text{Cl}^-$  (see Chapter 4). 5) The 33 kD extrinsic polypeptide contains no histidine (Oh-oka, et al., 1986).

If D1 and D2 are assumed to form a heterologous dimer, there are three histidines at the carboxy-terminal ends of each protein (His 337 on D2 and His 332 and His 337 on D1) that would be close enough to each other to form a cluster (see Figs. 34 and 35). Although many of the His residues in the membrane-spanning helices of these proteins have been tentatively assigned to the binding of reaction center components (e.g., P680,  $\text{Q}_A$ , Fe, etc.; see Trebst and Draber, 1986), there have been no assignments yet proposed for the 8 His residues on D1 and D2 that extend

Figure 34. A model for the arrangement of the intra-thylakoid loops of the D1 protein in spinach. Only the inner portions of the five membrane-spanning helices are shown. The amino acid sequence and location of the loops is taken from Trebst and Draber (1986). The 5 amino acid ligands for the Mn "A" binding site are labeled A1, A2, etc. Those for the "B" site are labeled B1, B2, etc. "RAH" refers to a redox-active histidine. X1 and X2 are histidines suggested to be involved in Cl<sup>-</sup> binding; the third histidine is on D2 (see Fig. 35).

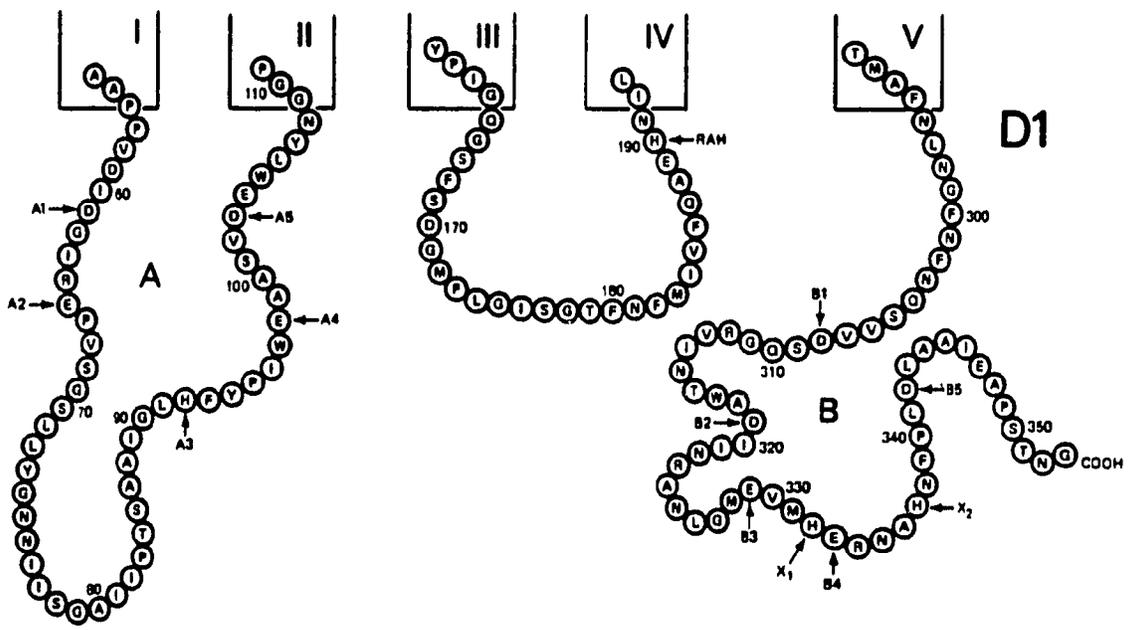
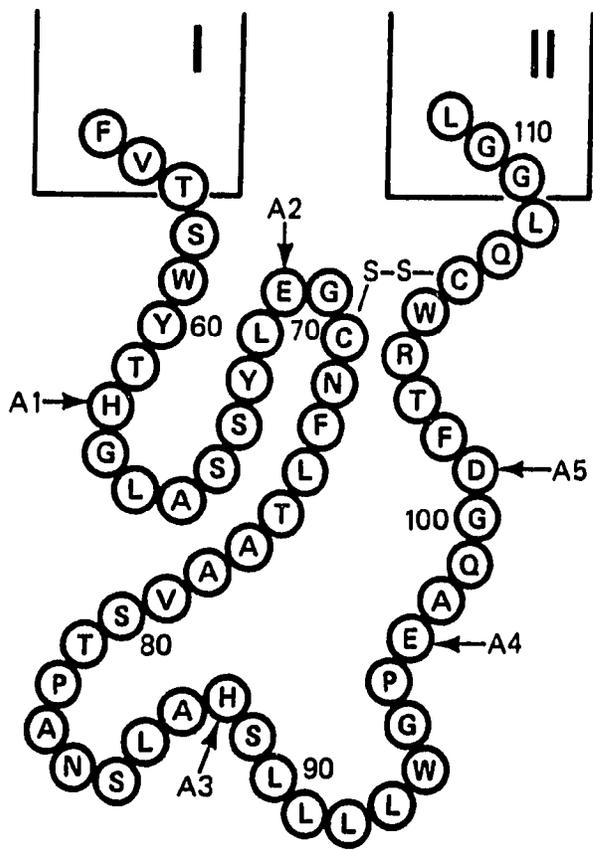


Figure 35. A model for the arrangement of the intra-thylakoid loops of the D2 protein in spinach. Only the inner portions of the five membrane-spanning helices are shown. The amino acid sequence is taken from Trebst and Draber (1986), but was checked against several others in order to correct discrepancies (e.g. at residues 329-332 and 347-348; see Alt et al., 1984; Holschuh et al., 1984; and also Rasmussen, 1984). The 5 amino acid ligands for the Mn "A" and "B" binding sites are labeled as in Fig. 34. The 4 amino acids for the Mn/Ca "C" binding site are labeled C1, C2, etc. "RAH" refers to a redox-active histidine. X1 is a histidine involved in Cl<sup>-</sup> binding; the two others on D1 are shown in Fig. 34.



Figure 36. A model for the arrangement of the amino acids at the Mn "A" site of the D2 protein, assuming a disulfide bridge between Cys-72 and Cys-106. A disulfide bridge between these residues is expected to create a much tighter structure for the Mn binding site.



into the aqueous phase of the lumen (Trebst and Draber, 1986). Since we have proposed that three of these His residues are involved in the  $\text{Cl}^-$  mechanism, we must explain the probable function of the five remaining histidines.

#### **E. Proposed Structure for the Mn Binding Sites in PS II**

Because the D2 protein has been strongly implicated in Mn binding (Metz and Bishop, 1980), we decided to examine the amino acid sequence for spinach D2 and D1, in order to see whether the intra-thylakoid loop regions of these proteins (described above) contain any clusters of amino acids that are capable of forming Mn binding sites.

Studies of Mn binding sites in proteins have shown that Mn prefers to be coordinated by the unprotonated carboxylate side chains of aspartyl or glutamyl residues and the unprotonated imidazole of histidyl residues. Either tetrahedral or octahedral geometry is possible (Hughes, 1981). The X-ray crystal structure for the Mn (III) superoxide dismutase from Thermus thermophilus suggests that the Mn is part of a trigonal bipyramidal (5-coordinate) complex with water and histidine as the axial ligands and two histidines and an aspartate as the other coplanar ligands (Stallings et al., 1985). This geometry assumes that the aspartate contributes only one oxygen atom. The Mn (II) in concanavalin A is also coordinated by a combination of one histidine, two aspartates, one glutamate, and two water molecules (Hardman and Ainsworth, 1972).

In searching for potential Mn ligands on D1 and D2, we assumed a simple octahedral geometry for Mn, with five protein ligands (one axial), and a sixth axial water molecule. The assumption that all of the Mn are coordinated by  $\text{H}_2\text{O}$  is based on the model of Kambara and

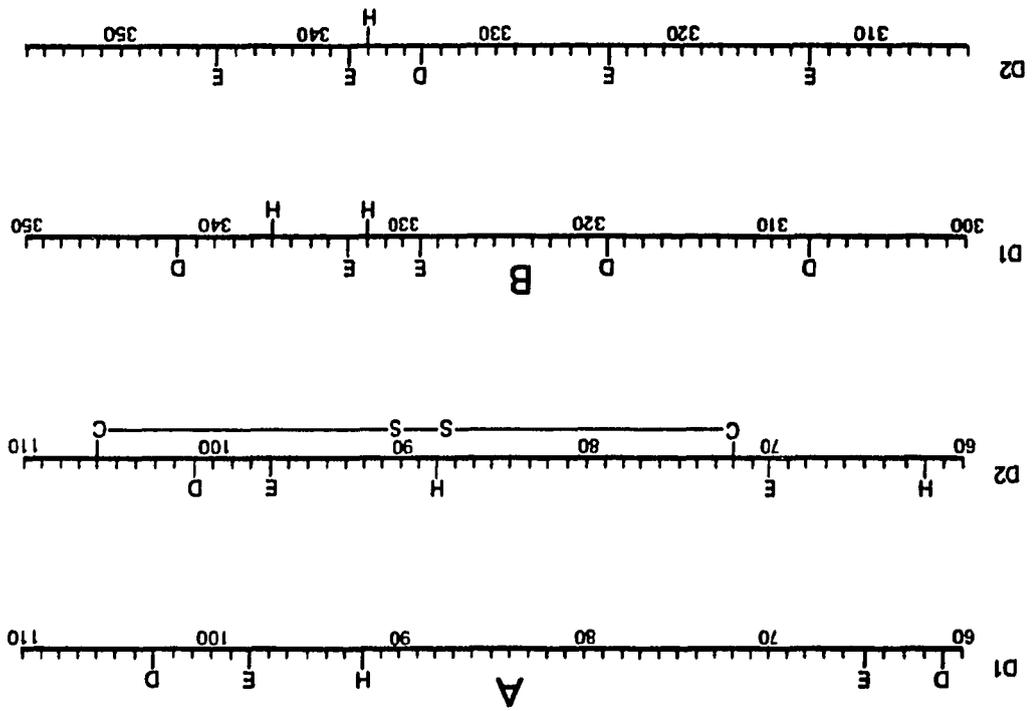
Govindjee (1985).

In order to narrow the search, we took advantage of the sequence comparison by Trebst and Draber (1986) of the spinach reaction center proteins D1 and D2 and the bacterial reaction center proteins L and M from R. capsulatus. Since the latter organism does not evolve O<sub>2</sub>, we focused our search on regions of D1 and D2 that have low homology with their bacterial counterparts, or that appear to be "insertions" into the L and M sequences.

Examination of the intra-thylakoid loop regions of D1 and D2 reveals that there are four such regions, each of which contains enough potential Mn ligands to act as a Mn binding site. These regions consist of the residues between the membrane-spanning helices I and II on both proteins (labeled "A" in Figs. 34 and 35) and the COOH-terminal portions of both proteins (labeled as "B" in D1, Fig. 34 and "B" and "C" in D2, Fig. 35). The D2-A site is consistent with possible Mn ligation, based on the presence of five potential ligands (2 His, 2 Glu, and 1 Asp). These residues are labeled A1-A5 in Figures 34-36. Because there are more than five potential ligands at the D1-A site, we aligned the sequence of this site with the sequence of the D2-A site (Fig. 37 A). The five D1 residues labeled A1-A5 (1 His, 2 Glu, 2 Asp) in Fig. 34 provide the best overall match to the D2-A site. The two sites are similar except for the substitution of D1-Asp 61 for D2-His 62. The tighter spacing in the D2-A site may explain the presence of a possible disulfide bridge.

Upon examining the regions labeled "B" in each protein (Figs. 34 and 35), we also noticed that there are two nearly identical sets of 5 carboxylate residues (4 Glu and 1 Asp on D2, and 2 Glu and 3 Asp on D1).

Figure 37. Amino acid sequence alignments for the Mn binding regions in D1 and D2. (A) Alignment of the Mn "A" sites. A potential disulfide bridge is shown for D2. (B) Alignment of the Mn "B" sites. Histidines proposed to be involved in Cl<sup>-</sup> binding are shown underneath each line.



These are labeled B1-B5 on each protein (Figs. 34 and 35). The sequence alignment (Fig. 37 B) shows that the spacing between potential ligands are quite similar. Although we favor the involvement of Histidines D1-332, D1-337, and D2-337 in the binding of  $\text{Cl}^-$ , we cannot rule out their involvement in Mn binding by this kind of analysis, particularly if they participate in ligand exchange. For example, D1-His 332 and D2-His 337 (see Fig. 37 B) could conceivably coordinate Mn in one conformational state of the OEC and bind  $\text{Cl}^-$  in another state by allowing D1-Glu 333 and D2-Glu 338 to replace them. Brudvig and Crabtree (1986) and Brudvig and de Paula (1987) have proposed that a conformational change in the structure of the active site Mn is necessary for water oxidation to occur. Simultaneous Mn and  $\text{Cl}^-$  binding at these two histidines is not likely, because protonation of the imidazoles would interfere with Mn coordination at the imidazole N-3 nitrogen.

In addition to the A and B sites, we also noticed that there is a dense cluster of carboxylates on D2 in the region labeled "C" (Fig. 35). The spacing of the non-adjacent carboxylate residues in this region (labeled as C1-C5 in Fig. 35) suggests that they might be grouped into a distorted rhombic or tetrahedral site. This kind of coordination has been suggested for Mn(II), based on the EPR properties of certain Mn(II)-substituted proteins (Haffner et al., 1974; see also Chapter 1).  $\text{Ca}^{2+}$  might also be coordinated at this site, although the amino acid sequence does not appear to match the sequence of any well-known  $\text{Ca}^{2+}$  binding sites. Calcium binding to such a site would perhaps explain the  $\text{Ca}^{2+}$  requirement for  $\text{O}_2$ -evolution in PS II membranes depleted of the extrinsic polypeptides.

On the other hand, the existence of a site containing a transition metal in a distorted rhombic environment could partly explain the

observation of a low-field ( $g=4.1$ ) EPR signal arising from the water-oxidizing site (for a review, see Dismukes, 1986), although it is not certain whether such a site would give rise to the flash-induced component of the  $g=4.1$  signal. de Paula et al. (1986) have attributed the  $g=4.1$  signal to the tetranuclear Mn, based on measurements of its temperature dependence; however, because of the anomalous behavior of this signal compared to the  $S_2$  multi-line EPR signal, Aasa et al. (1987) have postulated that it may arise from monomeric Mn(IV). By using Q-band EPR, Mavankal et al. (1987) have reported the presence of protein-bound Mn(II) in PS II membranes lacking the extrinsic polypeptides.

Because of their relative isolation from the other histidines and their close proximity to the proposed binding sites for the reaction center components in helices III and IV (Trebst and Draber, 1986), we propose that D1-His 190 and D2-His 190 are involved in creating an electron-conducting pathway from the Mn sites in the lumen to the component Z, the donor to P680 (see Chapter 1). This assignment is an extension of previous models that have proposed a role for histidine as a redox-active ligand (RAL) between Mn and Z (Kamabara and Govindjee, 1985; Padhye et al., 1986; comparable to the A-site histidines in our model). By analogy, we have labeled each of these residues as a redox-active histidine (RAH).

The above analysis has included all of the histidines believed to reside on the intra-thylakoid domains, but has not accounted for all of the carboxylate residues. Some of these carboxylates, however, could be involved in salt-bridges with positively-charged residues at equivalent locations on the opposite protein. For example, it is possible that D1-Glu 104 pairs with D2-Arg 104 to enable the two proteins to remain

linked together. Residue D1-Glu 347 was not included because it is not found in Chlamydomonas (Erickson et al., 1983).

Comparison of D1/D2 sequences from various organisms reveals that the D1 A- and B-sites are completely conserved (residues and spacing) among chlamydomonas, spinach, tobacco, and soybean (Zurawski et al., 1982; Erickson et al., 1983; Spielman and Stutz, 1983; Alt et al., 1984; Holschuh et al., 1984). The D2 B-site is conserved between spinach and pea (Rasmussen et al., 1984). In chlamydomonas, however, the sequence homology with spinach disappears at D2-B Glu 313, the first ligand of the B-site (Rochaix et al., 1984). The chlamydomonas sequence does, however, contain a histidine in this region. The D2 C-site is conserved in spinach, pea, and chlamydomonas (Alt et al., 1984; Holschuh et al., 1984; Rasmussen et al., 1984; Rochaix et al., 1984). Comparison with the bacterial L and M subunits indicates that these sites are not present in bacteria (see Trebst and Draber, 1986).

Figure 38 provides a schematic illustration of how Mn might be coordinated at each of these sites. Based on the results of EPR studies of the  $S_2$  state (see Chapter 1), it has been proposed that the tetranuclear Mn active site contains 3 Mn(III) and 1 Mn(IV) (Dismukes and Siderer, 1981). Studies of the Tris-sensitivity of the  $S_2$  state also indicates that one of the four Mn atoms is uniquely sensitive to this inhibitor (Yocum et al., 1981). The relatively small size of the D2-A site (consistent with the coordination of a smaller, more highly charged Mn atom), as well as its unique coordination by two imidazoles, make it an appropriate candidate for the Mn(IV) site in  $S_2$ .

We have summarized our ideas for the Mn and  $Cl^-$  sites into an overall model for the intrinsic portion of the OEC (Fig. 39). Many structural features of this model correspond to the predictions of

Figure 38. A schematic representation of the possible coordination geometry of Mn in the A, B, and C sites. The Mn valence states for the A- and B-sites represent those that have been proposed for the  $S_2$  state. See section E for a discussion of the possible role of histidine at the B-site. "M<sup>2+</sup>" represents a Mn<sup>2+</sup> or Ca<sup>2+</sup> ion bound to the C-site.

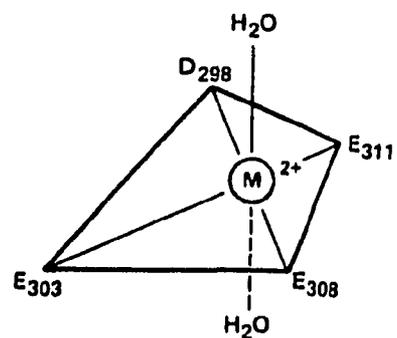
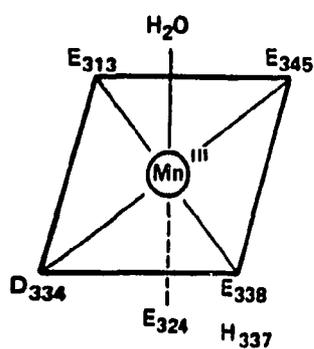
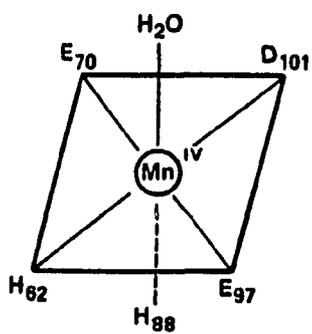
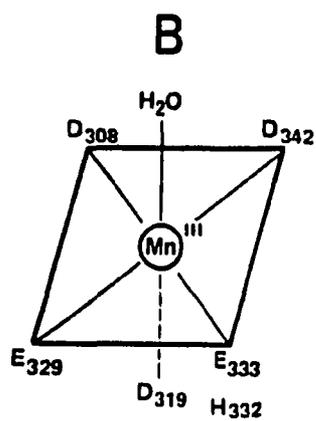
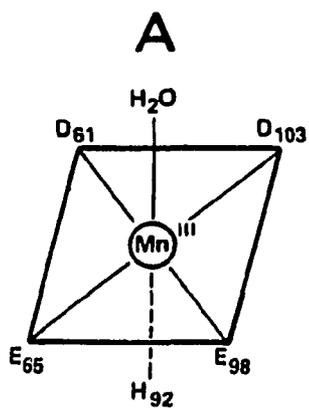
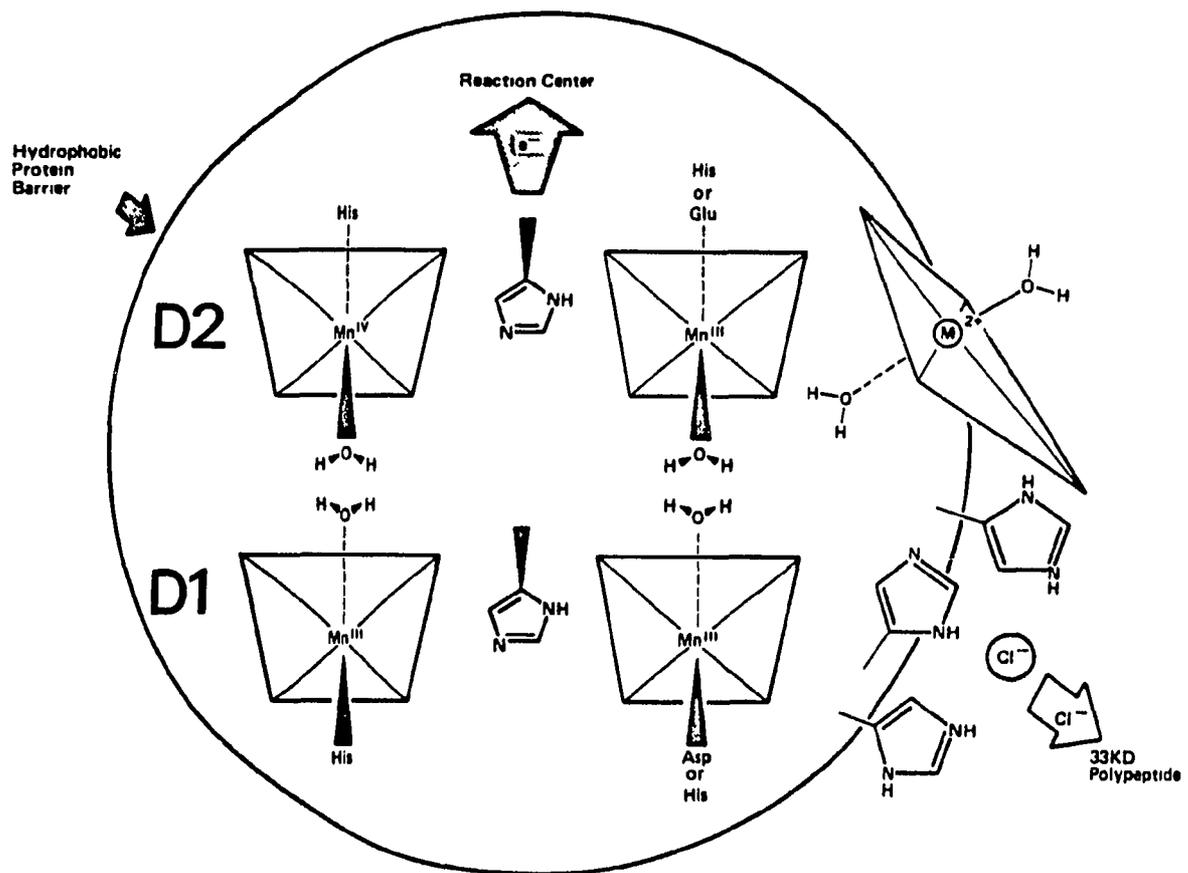


Figure 39. A model for the Mn active site and intrinsic Cl<sup>-</sup> binding site of the OEC. The four high-valence Mn sites are depicted as a tetranuclear cluster. The hydrophobic A-sites are on the left and the hydrophilic B-sites are on the right (see section E for a discussion of the possible role of histidine at the B-site). The Mn valence states represent those that have been proposed for the S<sub>2</sub> state of the OEC. No H<sub>2</sub>O chemistry is shown. The monomeric Mn/Ca site (M<sup>2+</sup>) connects the active site to the aqueous phase by facilitating the transfer of H<sub>2</sub>O. The two imidazole groups in the center represent D1/D2-His 190, which may be involved as redox-active groups in the transfer of electrons to Z. The three imidazole groups involved in the intrinsic Cl<sup>-</sup> binding site are shown to the lower right.



Kamabara and Govindjee (1985) that were derived to explain the function of the OEC Mn. The A-sites, for example, reside in a domain that is more hydrophobic than that of the B-sites. In addition, the B-sites appear to be associated with the proton-withdrawing mechanism that requires  $\text{Cl}^-$ . The coordination of the tetranuclear Mn in this scheme is also not inconsistent with the model of Brudvig and Crabtree (1986) and Brudvig and de Paula (1987), since some of the oxygen/hydroxo-ligands to Mn that are proposed in their models could be supplied by the carboxylate residues we have identified, or by  $\text{H}_2\text{O}$  bridges between the carboxyl groups and Mn. The function of the C-site metal atom in the present model might be to channel water molecules from the aqueous phase into the hydrophobic active site. Overall, this kind of arrangement creates an extended network of water molecules connected by hydrogen bonds (see Kambara and Govindjee, 1985). As the water molecules are oxidized at the Mn sites, the electrons are channeled upward to the reaction center, and the  $\text{H}^+$ s are moved out toward the extrinsic polypeptides in the lumen. The structure is therefore consistent with the process of charge separation as it relates to the splitting of water.

#### **F. Proposed Structure and Properties of the Extrinsic $\text{Cl}^-$ Binding Sites**

The appearance of linewidth maxima in the  $^{35}\text{Cl}$ -NMR binding curve for spinach thylakoids and PS II membranes led us to conclude that  $\text{Cl}^-$  binds to a number of sites (possibly as many as 11-12) that are sequestered from the bulk solvent in the absence of  $\text{Cl}^-$  (see Chapter 2). The model described earlier for the intrinsic  $\text{Cl}^-$  binding site (Fig. 32) required that  $\text{H}^+$  and  $\text{Cl}^-$  be transferred out of the active site region and onto the 33 kD extrinsic polypeptide. Both our  $^{35}\text{Cl}$ -NMR studies and

the work of other laboratories using  $O_2$ -evolution assays have indicated that  $Ca^{2+}$  is required for the proper functioning of the OEC in PS II membranes that have been treated with 1.0 M NaCl (see Chapter 4). Based on the results of our search for Mn binding sites on D1/D2, we decided to examine the 33 kD polypeptide for potential divalent metal sites. By so doing, we hoped to be able to organize the structure of the protein into functional domains, in order to identify possible  $Cl^-$  binding regions.

The assumption that we used for analyzing this protein was that it possessed large sites with octahedral geometry, wherein a  $Ca^{2+}$  or other similar divalent cation could be weakly coordinated by six protein ligands. When we arbitrarily divided the protein into 4 domains, each of about 60 amino acid residues, and aligned the sequences for the carboxylate residues (which, along with carbonyl groups, typically coordinate  $Ca^{2+}$  in proteins; Hughes, 1981), we quickly noticed that these four domains can be organized into two palindromic pairs (Fig. 40). Each member of the pair contains six carboxylate residues whose spacing is almost exactly reversed on the other domain. The two pairs are formed by residues 1-62 (Domain 1) and 187-246 (Domain 4) and by residues 62-121 (Domain 2) and 121-180 (Domain 3). This alignment implies a possible head-to-tail arrangement of the protein (see below).

Figure 41 shows how these domains might be organized into 4 octahedral sites. The apparent ligand overlap at Glu-62 and Glu-121 has a parallel in the divalent metal binding sites in Concanavalin A, where the carboxyl groups of residues Asp-10 and Asp-19 are shared between  $Mn^{2+}$  and  $Ca^{2+}$  (Hardman and Ainsworth, 1972). Figure 42 shows how this overlap might tend to orient the octahedral domains with respect to each

Figure 40. Sequence alignment for the carboxylate residues on four domains of the 33kD extrinsic polypeptide. Asterisks denote possible axial ligands in each octahedron. A possible disulfide bridge is shown for Domain 1.

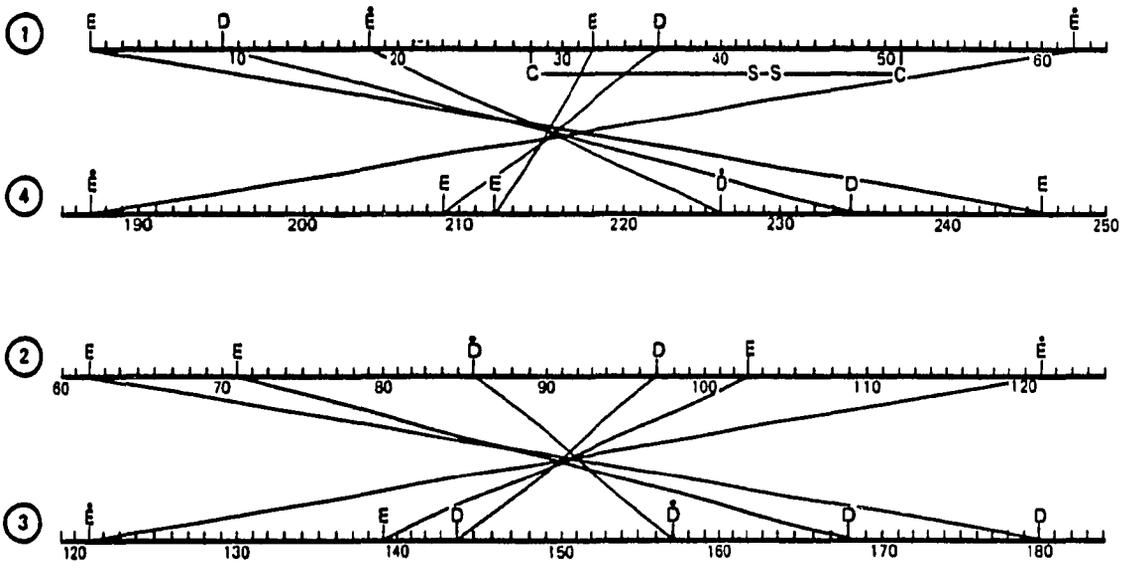


Figure 41. A schematic representation of the possible coordination geometry for four divalent metal binding sites on the 33 kD extrinsic polypeptide. Each octahedral site corresponds to one of the four protein domains.

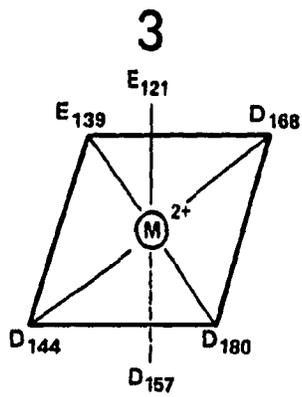
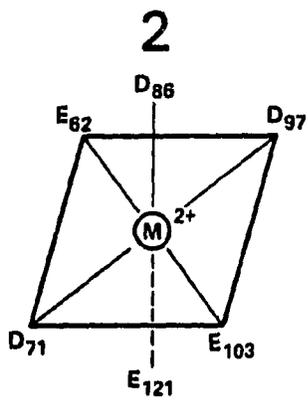
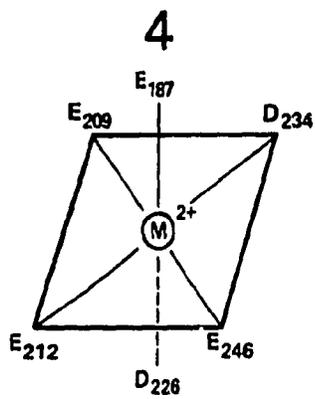
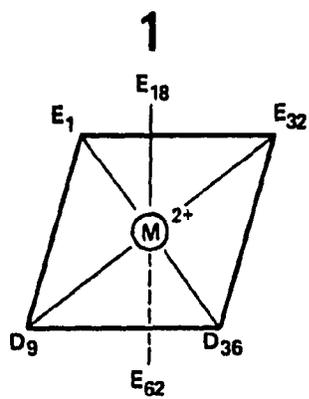
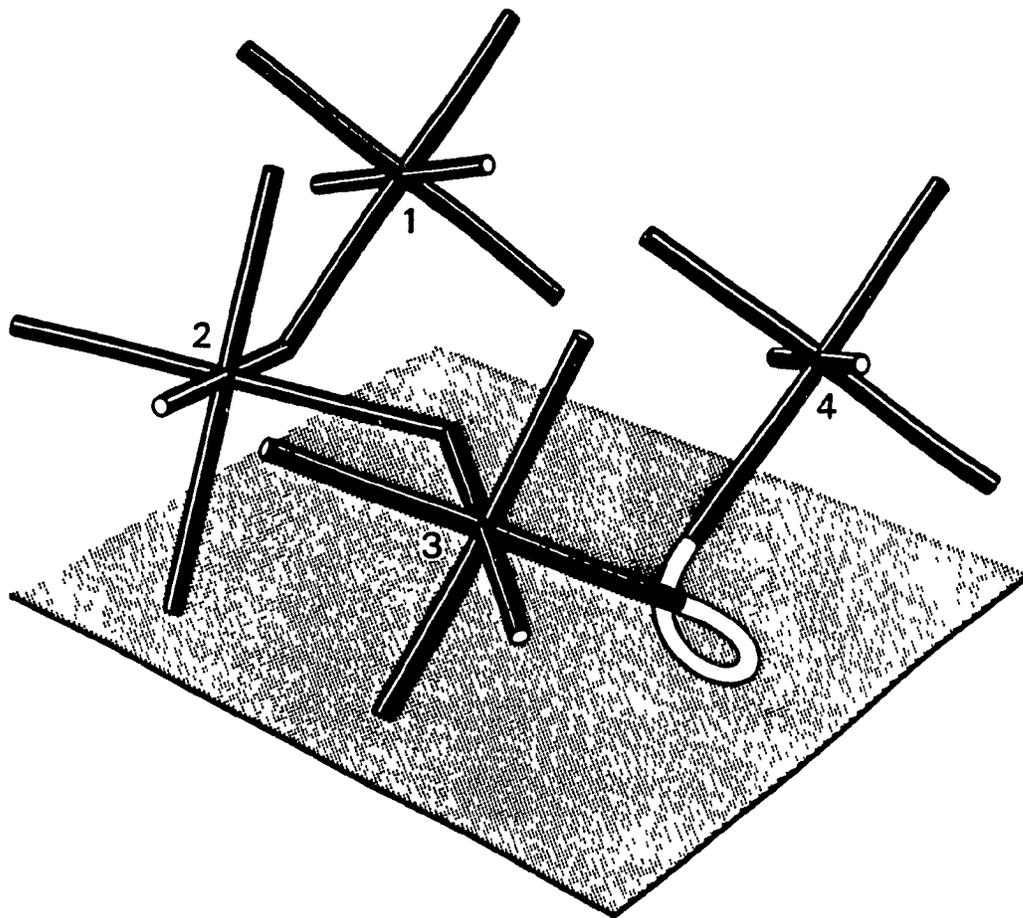


Figure 42. A schematic representation of the relative orientations of the four octahedral domains of the 33 kD extrinsic polypeptide. Amino acid ligands are at the vertices. Shared ligands occur at the interface between Domain 1 and Domain 2 and between Domain 2 and Domain 3.



other.

In comparison to the sizes of other well-known  $\text{Ca}^{2+}$  binding sites, the sites described here for the 33 kD polypeptide are relatively large, although thermolysin is known to have a site that spans 52 residues and coordinates a  $\text{Ca}^{2+}$  ion with five carboxylates and one water molecule (Levine and Williams, 1982). The size of the sites on the 33 kD polypeptide may be responsible for the apparently weak binding of  $\text{Ca}^{2+}$  in the absence of the 18 kD and 24 kD polypeptides.

It is important to realize that  $\text{Ca}^{2+}$  is not likely to be bound to these four domains in the native complex. Instead, these sites are probably occupied by portions of the 18 kD and 24 kD polypeptides. In this respect, they should perhaps be considered pseudo-binding sites, because they are only able to bind  $\text{Ca}^{2+}$  (weakly) when the 18 kD and 24 kD polypeptides have been removed.

Although this four-domain arrangement accounts for most of the carboxylate residues on the polypeptide, there are two small regions between residues 87-109 and 181-187 where the spacing of the carboxylates does not fit the overall pattern. In an earlier paper (Coleman et al., 1987), we noted that the amino acid sequence in these two regions showed partial homology with the sequences of some of the  $\text{Ca}^{2+}$  binding sites of troponin C and calmodulin. Upon further examination, we realized that although the longer sequence is partially homologous with a calmodulin/troponin C-type  $\text{Ca}^{2+}$  binding loop (Fig. 43), the shorter sequence (Fig. 44) shows better homology with the so-called acidic clusters of troponin C and calmodulin (Demaille, 1982). These clusters have been suggested to be involved in binding troponin I (in troponin C; Leavis, 1978) and in binding  $\text{Mg}^{2+}$  (see Milos et al., 1986, for calmodulin).

Figure 43. Amino acid sequence comparison between a region of the 33 kD extrinsic polypeptide (top) and a reconstructed "primordial"  $\text{Ca}^{2+}$  binding domain (bottom) for  $\text{Ca}^{2+}$  binding proteins (Demaille, 1982). Asterisks denote possible  $\text{Ca}^{2+}$  ligands on the 33 kD polypeptide.



Figure 44. Amino acid sequence comparison between an acidic cluster in the 33 kD extrinsic polypeptide (top line) and 4 similar sites in calmodulin (Milos et al., 1986).

181  
E E E L Q K E N N K  
9  
I A E F K E A F S L  
45  
E A E L Q D M I N E  
82  
E E E I R E A F R V  
118  
D E E V D E M I R E

A second acidic cluster occurs in the D1 protein at residues 241-245 (Zurawski et al. 1982), which Trebst and Draber (1986) place on the outer membrane surface. This D1 cluster sequence Q-E-E-E-T shows partial homology with the "ancestral" acidic cluster sequence Q-T-E-E-E for  $\text{Ca}^{2+}$  binding proteins (Demaille, 1982).

Although Fig. 43 shows that a number of potential  $\text{Ca}^{2+}$  binding groups may be present among residues 87-94 and 100-109 of the 33 kD polypeptide, it is not certain from this analysis whether these groups would be able to constitute a relatively high-affinity  $\text{Ca}^{2+}$  binding loop, such as those in troponin C and calmodulin. It is possible that the 18 kD and 24 kD polypeptides may contribute additional ligands (see discussion in Chapter 4) or that water molecules are involved in coordination. A somewhat stronger case can be made for a high-affinity  $\text{Ca}^{2+}$  binding loop within residues 223-232 of D2 (Fig. 45), a sequence which Trebst and Draber (1986) locate on the outer surface of the membrane. This sequence shows a greater homology with one of the  $\text{Ca}^{2+}$  sites in calmodulin (Demaille, 1982; Grand, 1985). The presence of two high-affinity  $\text{Ca}^{2+}$  binding sites within PS II is consistent with recent experimental evidence (Cammarata and Cheniae, 1987; Katoh et al., 1987; Miller et al., 1987).

The apparent homology between regions of the D1/D2 proteins or the 33 kD polypeptide and calmodulin/troponin C may have functional significance for photosynthesis. Both calmodulin and troponin C activate  $\text{Ca}^{2+}/\text{Mg}^{2+}$  ATPases (Waisman et al., 1981; Potter and Johnson, 1982; Grand, 1985), and the chloroplast ATPase belongs to this same general class (McCarty and Carmeli, 1982).

Assignment of the carboxylate residues to the low-affinity and high-affinity  $\text{Ca}^{2+}$  sites and the acidic cluster leaves several of the

Figure 45. Amino acid sequence comparison between a region of the D2 protein (top) and a sequence for a  $\text{Ca}^{2+}$  binding region in calmodulin (bottom). Asterisks denote possible  $\text{Ca}^{2+}$  ligands on the D2 protein. The numbering sequence for calmodulin is taken from Grand (1980).



carboxylates unaccounted for. We have already proposed (see Chapters 2 and 3) that the  $^{35}\text{Cl}$ -NMR linewidth maxima arise from  $\text{Cl}^-$  binding to positively-charged residues (i.e. Lys or Arg) on the 33 kD polypeptide, and that these residues might be salt-bridged to negatively-charged residues in the  $\text{Cl}^-$  depleted complex. With this in mind, we examined whether the remaining acidic groups (Asp, Glu, and Tyr) could be paired in an orderly way with the Lys and Arg residues. Fig. 46 shows that the (+) and (-) charges can be arranged in pairs that connect domains 1 and 4 and domains 2 and 3 in a palindromic manner. The positive charges in these salt-bridges are distributed as follows: 5 on domain 4, 3 on domain 1, 2 on domain 3, and 1 on domain 2. This distribution of the sequestered  $\text{Cl}^-$  binding groups among 4 domains was predicted by the  $^{35}\text{Cl}$ -NMR results in Chapter 1.

A possible mechanism for  $\text{Cl}^-$  binding at these sites (based on earlier models by Homann et al., 1983; Coleman and Govindjee, 1985) is presented in Fig. 47. This mechanism proposes that  $\text{Cl}^-$  ions destabilize the salt-bridges between the domains by binding to the positive charges. This binding would be expected to raise the  $\text{pK}_a$ 's of the carboxylate or phenolic groups (Mehler and Eichele, 1984) and thereby stimulate them to accept protons from the intrinsic  $\text{Cl}^-$  site described above (see Scheiner et al., 1985). We propose that the salt bridges rupture in a defined sequence, first exposing 5  $\text{Cl}^-$  binding sites on Domain 4, then 3 sites on Domain 1, 2 sites on Domain 3, and 1 site on Domain 2. Protons simultaneously bind to the negative charges on the complementary domains, thereby accelerating the rate of  $\text{O}_2$ -evolution.

Figure 46. Sequence alignment for the acidic amino acid residues not involved in metal binding and the positively-charged basic residues on the 33 kD extrinsic polypeptide. Asterisks denote positively-charged residues involved in salt-bridges. These residues may represent sequestered Cl<sup>-</sup> binding sites that are distributed asymmetrically among the four domains.

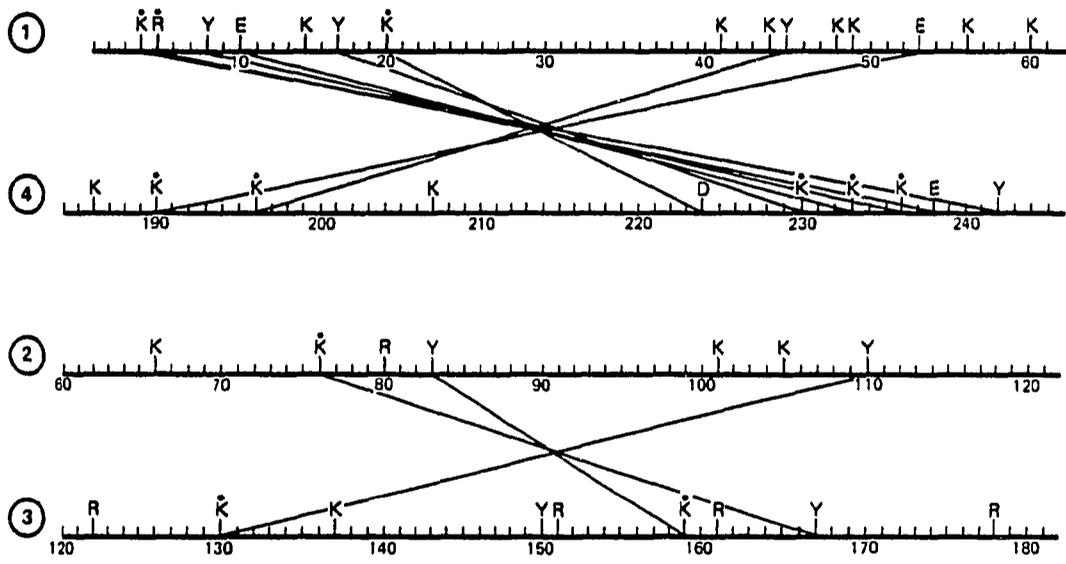


Figure 47. A model for the mechanism of the extrinsic  $\text{Cl}^-$  binding sites on the 33 kD polypeptide.  $\text{Cl}^-$  and  $\text{H}^+$  transferred from the intrinsic sites (Fig. 32) break the salt-bridges between the four domains of the polypeptide in an ordered sequence as the  $[\text{Cl}^-]$  increases.  $\text{Cl}^-$  binds to Lys and Arg;  $\text{H}^+$  binds to Asp, Glu, and Tyr (see Fig. 46). The open rectangle on Domain 2 represents a possible high-affinity  $\text{Ca}^{2+}$  binding site. The open rectangle between Domains 3 and 4 represents the acidic cluster Glu 181-Glu 183. The  $\text{Cl}^-/\text{H}^+$  binding sequence begins with all of the salt-bridges intact in the  $\text{Cl}^-$  depleted complex (A). When  $\text{Cl}^-$  and  $\text{H}^+$  are donated to the 33 kD polypeptide from the intrinsic  $\text{Cl}^-$  binding sites (see Fig. 32), they bind first to a group of 5 paired residues at the interface between Domains 1 and 4 (B). The other groups of salt-bridges are then broken sequentially as the  $\text{Cl}^-/\text{H}^+$  concentration rises. Thus,  $\text{Cl}^-$  binding sites open up in the following order: 5 on Domain 4 (B), 3 on Domain 1 (C), 2 on Domain 3 (D) and 1 on Domain 2 (E).  $\text{Cl}^-$  binding to these extrinsic sites may be responsible for sharp concentration-dependent increases in the  $^{35}\text{Cl}$ -NMR linewidth and "bursts" in the rate of  $\text{O}_2$ -evolution.



## G. Justification for the Mechanism at the Extrinsic Sites

This model for the extrinsic  $\text{Cl}^-$  binding sites explains the following observations: 1) The requirement of the 33kD polypeptide for high enzyme activity. 2) The high  $\text{Ca}^{2+}$  requirement for  $\text{O}_2$ -evolution and the appearance of the  $^{35}\text{Cl}$ -NMR linewidth maxima in the absence of the 18 kD and 24 kD polypeptides. The four octahedral domains are too large for tight  $\text{Ca}^{2+}$  binding, but neutralization of their negative charges may be necessary for the protein to attain an active conformation. 3) The presence of a high-affinity  $\text{Ca}^{2+}$  site in the reconstituted complex (see Ghanotakis et al., 1984). This site may occur in the region Glu-97 to Asp-109. 4) The observation that removal of  $\text{Cl}^-$  from the membrane requires deprotonation of strongly basic groups ( $\text{pK}_a > 8$ ). Lys and Arg are both involved in binding  $\text{Cl}^-$  at the extrinsic sites, and both have high  $\text{pK}_a$ 's. 5) The prediction that the OEC contains four sequestered  $\text{H}^+/\text{Cl}^-$  binding domains, which contribute to sharp increases in  $^{35}\text{Cl}$ -NMR linewidth and  $\text{O}_2$ -evolution (at low light intensity) in the ratio 5:3:2:1. 6) The apparent negative cooperativity with respect to the  $[\text{Cl}^-]$  in the  $\text{Cl}^-$  activation curve for Hill activity at low light intensity (see Chapter 1). As the  $[\text{Cl}^-]$  increases, the number of sites available for  $\text{Cl}^-$  binding within each remaining domain decreases, and therefore the slope of the curve quickly flattens out.

Although another intrinsic PS II protein, CP 47, contains numerous His, Asp, and Glu residues (see Morris and Herrmann (1984) for the DNA sequence), we do not believe that this protein binds the tetranuclear Mn cluster, since these residues cannot be divided into four discrete groups with the appropriate ligand composition.

## H. Summary

We have proposed a hypothetical model for the structure of the  $\text{Cl}^-$ , Mn, and  $\text{Ca}^{2+}$  binding sites within the OEC and the functional interactions between these sites. The intrinsic portion is comprised of sections of the D1 and D2 reaction center proteins. We propose that this region contains a tetranuclear (catalytic) Mn cluster and a monomeric Mn/Ca binding site, as well as a cluster of three histidines that bind  $\text{Cl}^-$  in the active site. In the presence of  $\text{Cl}^-$ , these histidines facilitate the transfer of  $\text{H}^+$  and  $\text{Cl}^-$  to a set of extrinsic  $\text{H}^+/\text{Cl}^-$  binding sites on the 33 kD extrinsic polypeptide. Chloride and  $\text{H}^+$  bind to this protein by disrupting salt-bridges between two pairs of complementary domains. The function of the  $\text{Cl}^-$  sites is to accelerate the removal of  $\text{H}^+$  from water.  $\text{Ca}^{2+}$  plays an important role in this process by maintaining the structure of both the intrinsic and extrinsic sites.

Overall, our model for the intrinsic and extrinsic  $\text{Cl}^-$  binding sites explains most of the  $^{35}\text{Cl}$ -NMR data presented in this thesis, and is consistent with the known involvement of both  $\text{Ca}^{2+}$  and several of the OEC polypeptides in the  $\text{Cl}^-$  effect. It also indicates that a high degree of cooperation between the intrinsic and extrinsic portions of the OEC is required for the process of photosynthetic oxygen evolution.

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## VI. SUMMARY

Chloride is required for the light-driven oxidation of water in the oxygen-evolving complex (OEC) of Photosystem II (PS II). Although much is known in general about the kinetics of activation and about the polypeptides involved in the effect, little is known in detail about: 1) the number and location of the  $\text{Cl}^-$  binding sites, 2) the  $\text{Cl}^-$  binding mechanism, and 3) the relationship between  $\text{Cl}^-$  binding, activation of  $\text{O}_2$ -evolution, and the structure of the OEC.

The objective of this thesis was to study directly the  $\text{Cl}^-$  binding mechanism by  $^{35}\text{Cl}$ -NMR and to relate this mechanism to the steady-state kinetics of oxygen-evolution. These measurements have provided new information about the number and location of the  $\text{Cl}^-$  binding sites, the involvement of  $\text{H}^+$ ,  $\text{Ca}^{2+}$ , and OEC polypeptides, and the effect of conformational changes on the dynamics of  $\text{Cl}^-$  binding. Based on these results, a model was devised to explain the action of  $\text{Cl}^-$  at two key sites within the OEC.

The initial study involved defining the effects of  $\text{Cl}^-$  on the intact complex. When  $\text{O}_2$ -evolution is measured in  $\text{Cl}^-$  depleted spinach thylakoids at low light intensity (5% of saturating) as a function of  $[\text{Cl}^-]$ , the system displays kinetic cooperativity with respect to  $\text{Cl}^-$  in the form of three intermediary plateaus in the curve. Similarly, measurement of the excess  $^{35}\text{Cl}$ -NMR linewidth (indicative of  $\text{Cl}^-$  binding) as a function of  $[\text{Cl}^-]$  in dark-adapted thylakoids shows an overall hyperbolic decrease which is interrupted by sharp increases in linewidth (linewidth maxima) at 0.3 mM, 0.75 mM, 3.25 mM, and 7.0 mM  $\text{Cl}^-$ . The relative heights of the linewidth maxima are in the ratio 5:3:2:1. This effect is located within PS II, as evidenced by similar results from  $^{35}\text{Cl}$ -NMR measurements of PS II membranes.

The apparent cooperativity in the steady-state activation curve can be partially eliminated by brief pre-illumination of the thylakoids prior to adding  $\text{Cl}^-$ . These results are best explained by proposing that  $\text{Cl}^-$  binds to four sequestered (salt-bridged) domains within the OEC, and that these domains contain a total of 11-12 individual sites. Binding of  $\text{Cl}^-$  is facilitated by the presence of  $\text{H}^+$  and vice versa. The pH-dependence of the excess  $^{35}\text{Cl}$ -NMR linewidth shows that  $\text{Cl}^-$  binding has a maximum at pH 6.0 and two smaller maxima at pH 5.4 and 6.5, which suggests that three groups (perhaps histidine) with  $\text{pK}_a$ 's in this region control the binding. The  $\text{Cl}^-$  binding observed by  $^{35}\text{Cl}$ -NMR can be partly eliminated by competition with 0.1 mM NaBr.

The effect of  $\text{Cl}^-$  on the structure and stability of the OEC was examined by thermal inactivation experiments. It was discovered that  $\text{Cl}^-$  depletion of thylakoids significantly increases the sensitivity of the OEC to mild heat treatment, in proportion to the degree of apparent depletion. Chloride-depleted thylakoids heated at  $38^\circ\text{C}$  for 3 min no longer show any cooperativity in either the steady-state kinetics of  $\text{Cl}^-$  activation or in the  $^{35}\text{Cl}$ -NMR binding curve. Heat-treated PS II membranes also show no linewidth maxima in the  $^{35}\text{Cl}$ -NMR binding curve. Although the Hill activity in partially  $\text{Cl}^-$  depleted thylakoids is very sensitive to  $[\text{Cl}^-]$  at temperatures below  $38^\circ\text{C}$ , the release of the catalytic Mn from the membranes is only observed at higher temperatures. The heating results indicate that  $\text{Cl}^-$  binds to highly-structured protein sites within the OEC and stabilizes their conformation.

Studies of PS II membranes inhibited by hydroxylamine, Tris, and salt-washing were undertaken to investigate in more detail the role of Mn and the OEC polypeptides in the  $\text{Cl}^-$  effect. Hydroxylamine treatment

(1.5 mM) of PS II particles, which removes Mn and abolishes oxygen evolution, affects the  $^{35}\text{Cl}$ -NMR binding curve only slightly, indicating that Mn is probably not directly required for  $\text{Cl}^-$  binding. On the other hand, washing with 0.8 M Tris, which removes the Mn and extrinsic polypeptides by denaturing the complex, eliminates almost all of the specific  $\text{Cl}^-$  binding observable by NMR.

Removal of the extrinsic 18 kD and 24 kD polypeptides by washing with 1.0 M NaCl not only reduces the overall  $^{35}\text{Cl}$ -NMR linewidth of the binding curve, but also gives rise to sharp decreases in linewidth at 0.3 mM and 0.75–0.9 mM  $\text{Cl}^-$ . Addition of 2.0 mM  $\text{CaSO}_4$  to these samples partially restores both  $\text{O}_2$ -evolution and the  $^{35}\text{Cl}$ -NMR linewidth maxima. This effect indicates that although the 18 kD and 24 kD polypeptides are not absolutely required for  $\text{Cl}^-$  binding, their role can only be duplicated by very high concentrations of  $\text{Ca}^{2+}$ . In NaCl-treated membranes in which the activity was partially restored by dilution of the incubation medium, the  $^{35}\text{Cl}$ -NMR linewidth maxima are also partially restored, indicating a reversible effect.

When all of the extrinsic polypeptides are removed by washing with 1.0 M  $\text{CaCl}_2$ , the  $^{35}\text{Cl}$ -NMR binding curve is significantly changed. Low-affinity  $\text{Cl}^-$  binding is still observable, but there is only a single, broad linewidth maximum centered at 0.5 mM  $\text{Cl}^-$ . This result, when considered in conjunction with the results of Tris- and NaCl-washing, indicates that although the remaining intrinsic proteins (e.g. D1/ D2) bind  $\text{Cl}^-$ , the native chloride-binding mechanism requires the added presence of the 33 kD polypeptide (in combination with the 18/24 kD polypeptides and/or  $\text{Ca}^{2+}$ ).

A working hypothesis was constructed to explain the mechanism of  $\text{Cl}^-$  activation of the OEC. According to this mechanism,  $\text{Cl}^-$  activates

O<sub>2</sub>-evolution by facilitating the removal of H<sup>+</sup> from water, a process that involves two types of sites. At the intrinsic site, Cl<sup>-</sup> is suggested to bind to a cluster of three histidines (formed by His-332 and 337 on the D1 protein and His-337 on the D2 protein) and to accelerate the abstraction of H<sup>+</sup> from water by raising the pK<sub>a</sub>'s of the imidazole groups. The presence of Cl<sup>-</sup> then enables this protonated site to donate H<sup>+</sup> and Cl<sup>-</sup> to the extrinsic sites. Chloride and H<sup>+</sup> donated from the intrinsic site attack specific salt bridges in the 33kD polypeptide, thereby opening up new binding sites on this protein. The Cl<sup>-</sup>-induced transfer of H<sup>+</sup> from the active site Mn to the extrinsic proteins stimulates the oxidation of water in this hypothesis. The model also proposes a structure for the active-site Mn on the reaction center D1 and D2 proteins. Here it is proposed that two pairs of catalytic Mn form a tetranuclear cluster, and that a fifth monomeric Mn or Ca ion directs the transfer of water molecules into the active site. Specific amino acid residues that are likely to participate as Mn ligands are identified on the luminal portion of the D1 and D2 proteins, based on their known coordination chemistry, their location within the protein chain, the regularity of their spacing, and their conservation through evolution. Finally, the model also identifies sites on both D2 and the 33 kD extrinsic polypeptide for possible high-affinity Ca<sup>2+</sup> binding.

In conclusion, these studies demonstrate that Cl<sup>-</sup> acts as a very dynamic activator, functioning throughout an extended region of the OEC. Chloride is suggested here to play a crucial role in directing the movement of positive charge through the hydrophilic regions of the complex, and in so doing, connects the intrinsic active site with the extrinsic polypeptides.

## VITA

William Coleman was born on August 17, 1957 in Cleveland, Ohio, where he received his primary and secondary education. In 1979, he received his B.A. degree cum laude in Biology and Literature from the University of Pennsylvania, where he was a Benjamin Franklin Scholar. He joined the University of Illinois in 1979, and has held teaching assistantships in the Departments of Chemistry and Plant Biology and research assistantships in the Department of Physiology and Biophysics. He has received three student travel awards from the American Society for Photobiology (of which he is a member) to present papers at their annual meetings, and has also received an NSF travel award to attend a NATO Summer School on Biomembranes (Spetsai, Greece, 1980). In 1984, he received the Francis and Harlie Clark Summer Research Grant from the School of Life Sciences at the University of Illinois. He is coauthor of the following publications:

1. The Effect of Chloride and Other Anions on the Thermal Inactivation of Oxygen Evolution, In: Advances in Photosynthesis Res. (C. Sybesma, ed.), Vol. 1, (1984) pp. 283-286, Martinus Nijhoff/Dr. W. Junk Publishers, Den Haag.
2. The Role of Chloride in Oxygen Evolution, In: Proceedings of the 16th FEBS Congress, Part B, (1985) pp. 21-28, VNU Science Press, The Netherlands.
3. The Electron Donor Side of Photosystem II: The Oxygen Evolving Complex, Photochem. Photobiol. 42 (1985) 187-210.
4. Applications of  $^{35}\text{Cl}$ -NMR to the Study of Chloride Binding in the Oxygen Evolving Complex of Photosystem II, In: Biomembranes: Structure, Biogenesis, and Transport (C. Rajamanickam and L. Packer, eds.), (1987) Today and Tomorrow's Printers, New Delhi, in press.

5. Involvement of  $\text{Ca}^{2+}$  in  $\text{Cl}^-$  Binding to the Oxygen Evolving Complex of Photosystem II, In: Progress in Photosynthesis Research (J. Biggins, ed.), Vol. 1, (1987) pp. 629-632, Martinus Nijhoff, Dordrecht, The Netherlands.