The Role of Chloride in Oxygen Evolution

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Summary

A brief review on the role of chloride in oxygen evolution is presented here. This paper deals with the discovery of the phenomenon, the site of action, specificity and effectiveness, possible binding sites and a possible mechanism of action. The phenomenon was discovered by Otto Warburg and has been studied extensively since the pioneering work of Sei Izawa and coworkers. The site of action is on the electron donor side of the Photosystem II (PSII) between the so-called water oxidation complex (WOC) and the electron donation sites of external donors such as NH_2OH, diphenylcarbazide and catechol. The effectiveness of the anions in supporting water oxidation, protecting O_2 evolution against mild heating and in preventing the dissociation of the extrinsic polypeptides of WOC, follows the series: $Cl^- > Br^- > NO_3^- > I^- > ClO_4^-$. Bicarbonate ($HCO_3^-$), that appears to be required on the electron acceptor side of PSII, is ineffective in replacing Cl^-. Sulphate (SO_4^{2-}) and PO_4^{3-} are totally ineffective, and F^- and OH^- might even be inhibitory. The size, ionic field and hydration energies of the anions seem to play significant roles in their effectiveness. It appears that several (4 to 40) Cl^- ions may be weakly and reversibly bound per WOC. In all likelihood, there are two binding domains: (1) intrinsic: on the PSII reaction centre proteins D_1 and D_2 (perhaps, on the lumenal histidines): and (2) extrinsic: on the extrinsic 33 kDa polypeptide (perhaps, on lysines and/or arginines). Removal of the extrinsic polypeptides seems to increase the [Cl^-] requirement for water oxidation activity. It is suggested that the intrinsic sites are the catalytic sites and the extrinsic sites are the regulatory sites. In the absence of Cl^-, the WOC is stuck in an abnormal state, and there is no O_2 evolution activity. Our hypothesis for Cl^- action is: it facilitates the abstraction of H^+s from H_2O during its oxidation to O_2 ($2H_2O = 4H^+ + e^- + O_2$). We have suggested that Cl^- binds to a positively charged amino acid (N^+) which is close to a negatively charged amino acid (B^-) in the neighbourhood of the substrate H_2O molecules. Binding to N^+ changes the pKa of B^- such that its affinity to H_2O protons increases. This facilitates H^+ abstraction from H_2O during its oxidation to O_2. In this hypothesis, release of H^+s is accompanied by the release of Cl^- ions.

I. Discovery

Forty-four years ago, Warburg and Lüttgens (1; also see 2) discovered that various anions (Cl^-, Br^-, I^- and NO_3^-) stimulate oxygen evolution during Hill reaction in water-washed broken chloroplasts. Chloride was the most effective anion, but rhodanide, SO_4^{2-} and PO_4^{3-} were ineffective in stimulating the Hill reaction. Gorham and Clendening (3) provided the first clear demonstration that Cl^- is specifically involved in O_2 evolution during the Hill reaction, and that the Cl^- effect is not just the reversal of injurious effects of exposure to light. Gorham and Clendenning further noted that (a) Cl^- shifts the pH optimum of the Hill reaction from 6.5 to 7.5; and (b) this Cl^- effect has similarity to that in dialyzed α-amylase. It is now generally accepted that Cl^- is a necessary cofactor for O_2 evolution, based mainly on the pioneering work of S. Izawa and coworkers [see e.g., Hind *et al.* (4); Izawa *et al.* (5); Kelley and Izawa (6); Muallem *et al.* (7) and Izawa *et al* (8)]. After 44 years of research, that occurred mostly in spurts, the molecular mechanism by which Cl^- activates O_2 evolution remains unknown. Readers are encouraged to consult earlier reviews by Homann *et al.* (9), Govindjee *et al.* (10, 11), Critchley (12), Homann (13), Coleman and Govindjee (14,15) and Coleman (16).

II. Site of Action

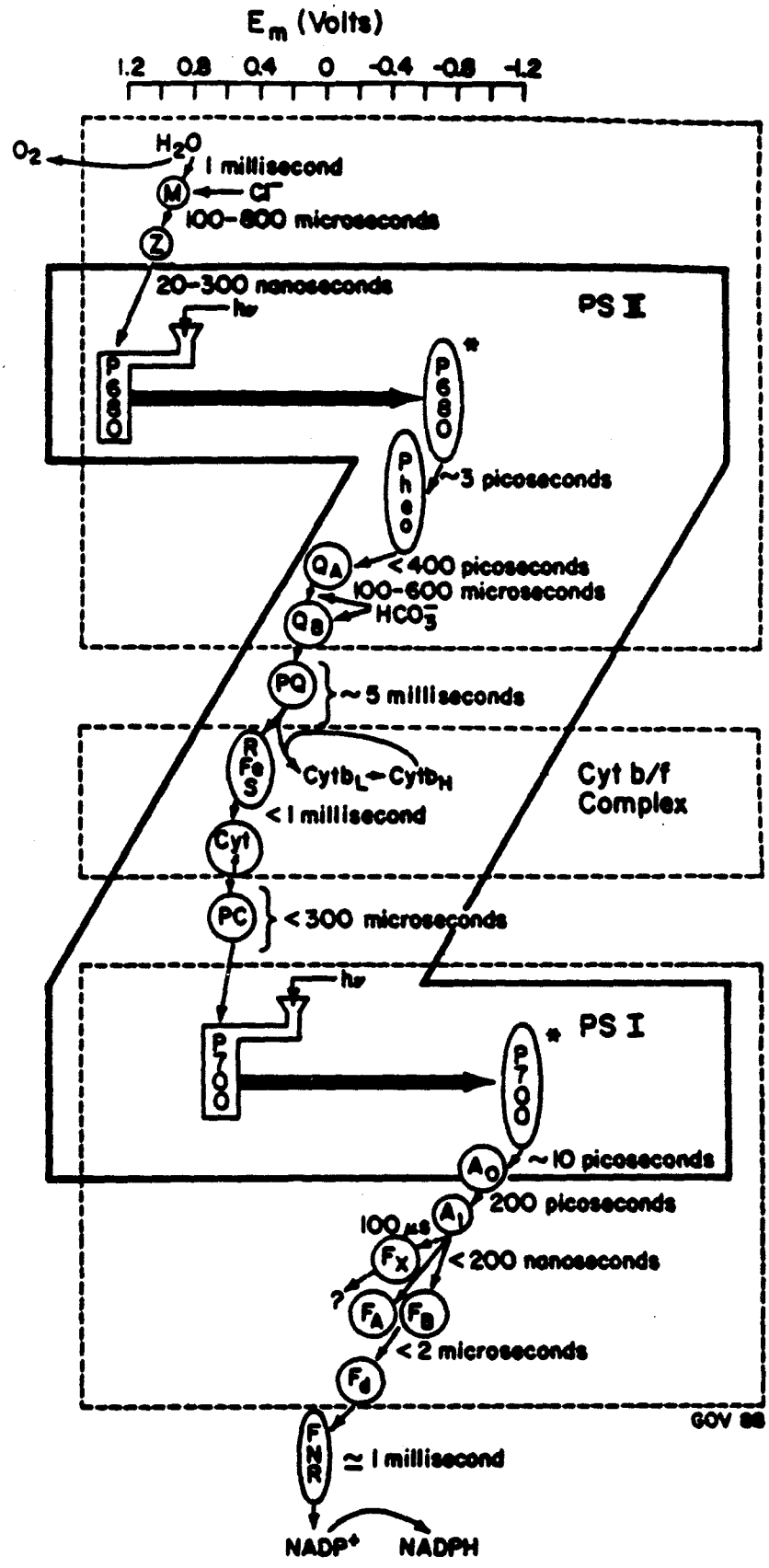
Figure 1 shows a current Z-scheme for electron flow from H_2O to $NADP^+$. This figure is a modification of that presented earlier by Govindjee and Eaton Rye (17); all the symbols are defined in the legend of the figure. The site of Cl^- action is shown here to be on the electron donor side of photosystem II (PSII) between water oxidation and the electron donation by Z of PSII (see left top of Fig 1); this site is suggested to involve the charge accumulator "M", of the water oxidation complex (WOC).

Figure 2 shows a current model of the thylakoid membrane with four major protein complexes: photosystem II, plastoquinol-plastocyanin oxidoreductase or cytochrome b_6/f complex, photosystem I, and the coupling factor (CF_1-CF_0) or ATPase; this figure is also a modification of that presented earlier (17). The site of Cl^- action is on the lumenal portion of photosystem II. The diagram shows Cl^- ions bound to the extrinsic 33 kilodalton polypeptides; Cl^- ions bound to the intrinsic polypeptides are not shown due to lack of space.

Figure 3 shows a scheme of the reaction centre of PSII, except that cytochrome b559 is not shown because its function is unknown. The reaction center of PSII was first isolated by Nanba and Satoh (25) and shown to contain two polypeptides of 32–34 kDa, lebelled as D_1 and D_2, and the two subunits of cytochrome b559 (4 and 9 kDa). This preparation contains 4 chlorophyll and 2 pheophytin molecules, but lacks plasto-

quinones Q_A and Q_B. The diagram shows the membrane portion of D_1 nd D_2 polypeptides arranged in the same fashion as the L and M polypeptides of bacterial reaction centres [see Michel and Deisenhofer (26)]; the lumenal portions of D_1 and D_2 as suggested by Coleman and Govindjee (15); the herbicide niche (see amino acids, as solid circles) as shown by Trebst (27); and the bicarbonate sites as suggested by Blubaugh and Govindjee (28) The *intrinsic* sites of chloride binding are suggested to be on histidines; specifically, H–337 on D_2 and H-332 and H-337 on D_1 are possible sites (15); there are no lysines on the lumenal portion of D_1 and and only one on D_2. Since 4Mn are involved in O_2 evolution, chloride binding sites may depend on where these manganese atoms are located. We expect the chloride site to be close to the Mn site. Coleman and Govindjee (15) proposed 4 possible Mn binding sites on the 4 lumenal loops; these are suggested to be bound to acidic amino acids, as shown in Fig. 3. Distances between Mn atoms larger than 3Å may be contradictory to the existing data [see e.g., reviews by Babcock (29), Brudvig (30) and Renger (31)]. If 'Z' is in fact tyrosine-160 (see Y on D_1-III in Fig. 2), it would be 'convenient' for the electron flow if the charge accumulator Mn were nearby. Thus, the possible sites of Mn could be on the lumenal portions connecting D_1-I and D_1-II (G. Brudvig, personal communication) and/or on the two loops with COOH ends (see D_1-V). Since the X-ray structure and the distances within the reaction centre of PSII are totally unknown, none of the possibilities can be ignored.

Bové *et al.* (32) showed that photophosphorylation associated with NADP-dependent non-cyclic electron flow, but not that associated with cyclic flow, requires chloride. Further localisation of the site of Cl^- action was made by chlorophyll *a* flourescence studies. Chlorophyll *a* fluorescence is an excellent monitor of PSII reactions (see reviews in 33). Upon illumination of a dark-adapted sample, fluorescence rises instantly to O (Fo) level, and then further increases to a 'P' (F maximum or Fp) level. This O→P rise is a monitor of the reduction of Q_A to Q_A^-. This fluorescence rise can be abolished either by stopping electron flow from the electron donor side of PSII, or by accelerating the electron flow out of Q_A^-, for example, by adding an efficient electron acceptor such as methylviologen. However, if the fluorescence rise can be restored by the addition of electron donors to Z or P680, such as hydroxylamine, diphenylcarbazide or catechol, then we know that the absence of a fluorescence rise was because of inhibition of electron flow from the electron donor side. Health and Hind (34, 35) and Critchley *et al.* (36 showed that chloride-deficient thylakoid membranes did not have any O→P fluorescence rise, but the addition of electron donors such as NH_2OH, catechol or diphenylcarbazide, or chloride (or bromide) restored the fluorescence rise (35,36; see Fig. 4). Thus, these data show that the chloride effect is on the electron donor side of PSII. Kelley and Izawa (6) showed restoration of electron flow in chloride-deficient thylakoid


E_m (Volts)
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O2
H2O
1 millisecond
M
Cl⁻
100-800 microseconds
Z
20-300 nanoseconds
hν
PS II
P680
P680*
Pheo
~3 picoseconds
QA
<400 picoseconds
100-600 microseconds
QB
HCO3⁻
PQ
~5 milliseconds
RFeS
Cytb_L → Cytb_H
<1 millisecond
Cyt b/f Complex
Cyt f
PC
<300 microseconds
hν
PS I
P700
P700*
A0
~10 picoseconds
A1
200 picoseconds
100 μs
FX
<200 nanoseconds
?
FA
FB
<2 microseconds
FD
FNR
≃ 1 millisecond
NADP+
NADPH
GOV 88

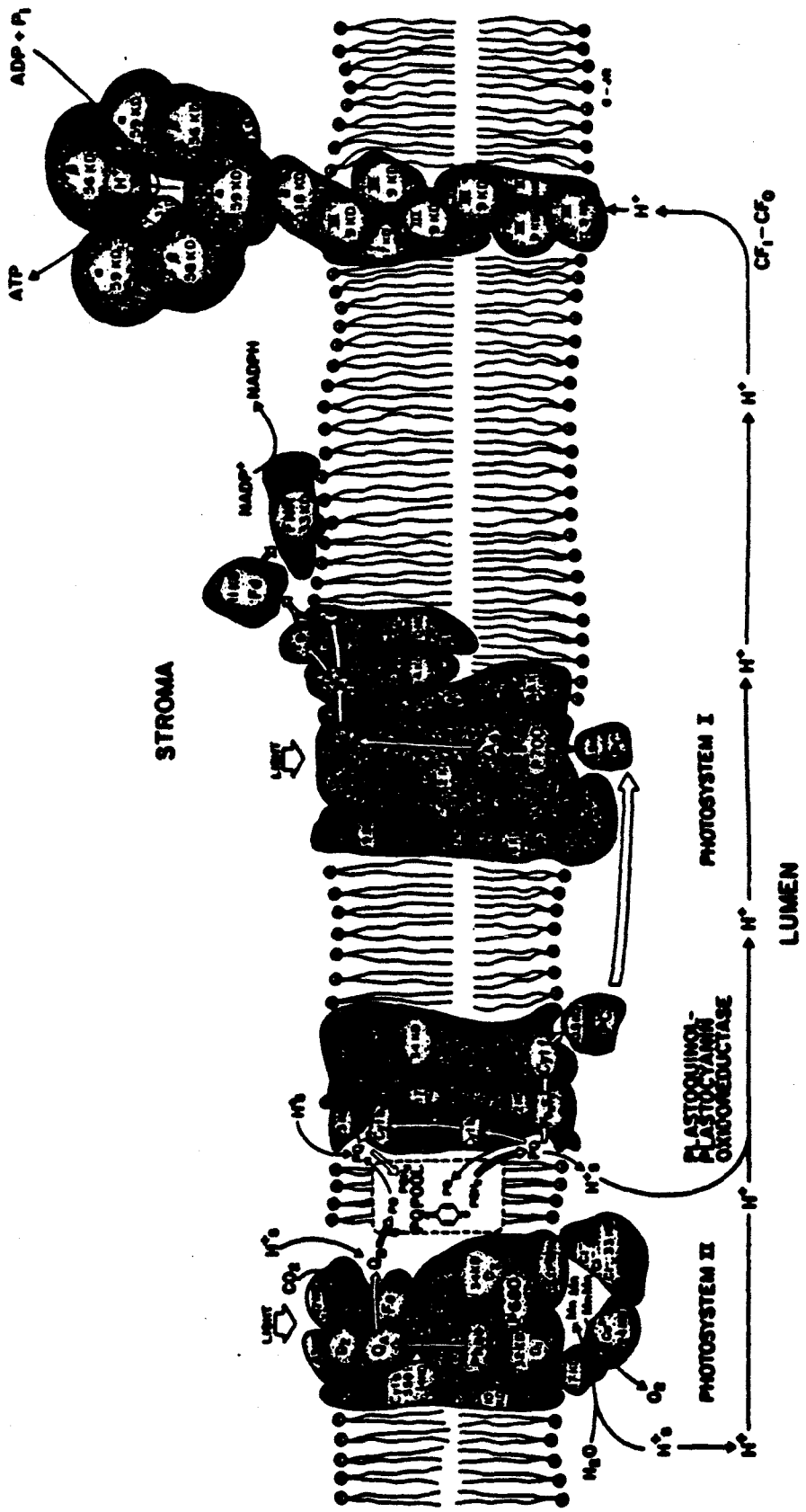
membranes by the addition of such electron donors or sufficient chloride, thus, establishing the site of chloride action.

III. Specificity and Effectiveness

The effectiveness of the anions in stimulating electron flow follows the series: $Cl^- > Br^- > NO_3^- > I^- > ClO_4^-$; F^- and OH^- are somewhat inhibitory, and SO_4^{2-} and PO_4^{3-} are ineffective. Sometimes F^- is found to be more effective than ClO_4^-, and NO_3^- is less effective than suggested

Fig. 1: *The 'Z' scheme of electron transport in photosynthesis*

Dashed retangles: Three major multiprotein complexes (PSII Photosystem II; Cyt b/f, cytochrome b_6/f complex; and PSI, Photosystem I), located in the thylakoid membrane, containing the photosynthetic components required for electron flow from H_2O to $NADP^+$. *Primary reactions:* The electron carriers are placed horizontally according to their midpoint redox potentials (Em, 7). Electron flow is initiated when a photon or exciton reaches the reaction centre chlorophyll *a* P680 (in PSII) and P700 (in PSI) (see hν going into the funnel). P680* and P700* (see ovals) indicate the first singlet excited states of P680 and P700. The *first* reaction of P680* is the conversion of excitonic energy into chemical energy *i.e.* charge separation, with the formation of the cation $P680^+$ and the anion pheophytin$^-$ ($Pheo^-$) within ~ 3ps. However, the first reaction of P700*, the charge separation into $P700^+A_o^-$, may occur in ~ 10ps. Here, A_o is a special chlorophyll *a* molecule. The $P680^+$ recovers its 'lost' electron from Z, now thought to be tyrosine-160 of the D_1 polypeptide of PSII. *Secondary reactions of PSII.* The positive charge on Z is then transferred to the charge accumulator 'M', or the water oxidizing complex (WOC). It is suggested that 'M' is nothing else but a Mn-cluster located on the lumenal portions of the D_1 and D_2 polypeptides of PSII (see Fig. 3). The *intrinsic* binding site of Cl^- lies somewhere in this region. Water oxidation seems to require another polypeptide, an extrinsic 33 kDa polypeptide, and, this is where *extrinsic* binding sites of Cl may exist. Four positive charges must accumulate before an O_2 molecule is evolved. The $Pheo^-$ delivers the extra electron to a primary (plastoquinone) electron acceptor Q_A located on the D_2 polypeptide of PSII; Q_A^- delivers its electron to a secondary (plastoquinone) electron acceptor Q_B, located on the D_1 polypeptide of PSII (see Fig. 3). Bicarbonate ions seem to be involved in the Q_A–Fe–Q_B region, where Fe is an iron atom (Fe^{2+}) between Q_A and Q_B. After reduction to plastoquinol, that is after two turnovers of the reaction centre P680, Q_B (H_2) exchanges with a mobile plastoquinone (PQ) molecule. *Reactions of the Cyt b/f complex.* Plastoquinol (PQH_2) delivers one electron to the Rieske Fe-S centre (R-Fe-S), and the other to a cytochrome b (b_L). The electron on R-Fe-S reduces cytochrome f(Cyt f) and the one on $Cytb_L$ is transferred to Cytochrome b_H (Cyt b_H), returning in a cyclic process (called the 'Q'-cycle).
Secondary reactions of PSI. Reduced Cyt f delivers its extra electron to a copper protein plastocyanin (PC) which delivers the electron to $P700^+$ (produced in the PSI primary reaction). On the other hand, A_o passes its electron to A_1 (perhaps, a phylloquinone). The rest of the electron carriers are: F_x (an iron-sulphur centre X), F_B (an iron-sulphur centre B), F_A (an iron-sulphur centre A), Fd (ferredoxin) and FNR (Ferredoxin-$NADP^+$ reductase). *Reaction times.* The diagram shows either measured or estimated times of the various reactions in the Z-scheme, except for the production of P680* and P700* that occur on a femtosecond time scale. The bottleneck reaction is of the order of 5 ms and it involves the total time involved in the exchange of $Q_B(H_2)$ with PQ, diffusion of PQH_2 to Cyt b/f complex, and the reoxidation time of PQH_2. The electron flow from A_0 to A_1 may occur in 50 ps, rather than 200 ps. (The diagram does not show the steps involved in H^- uptake and release.)


ADP + Pi
ATP
NADP+
NADPH
STROMA
LUMEN
PHOTOSYSTEM I
PHOTOSYSTEM II
PLASTOQUINOL-PLASTOCYANIN OXIDOREDUCTASE
H+
CF1 – CF0
O2
H2O

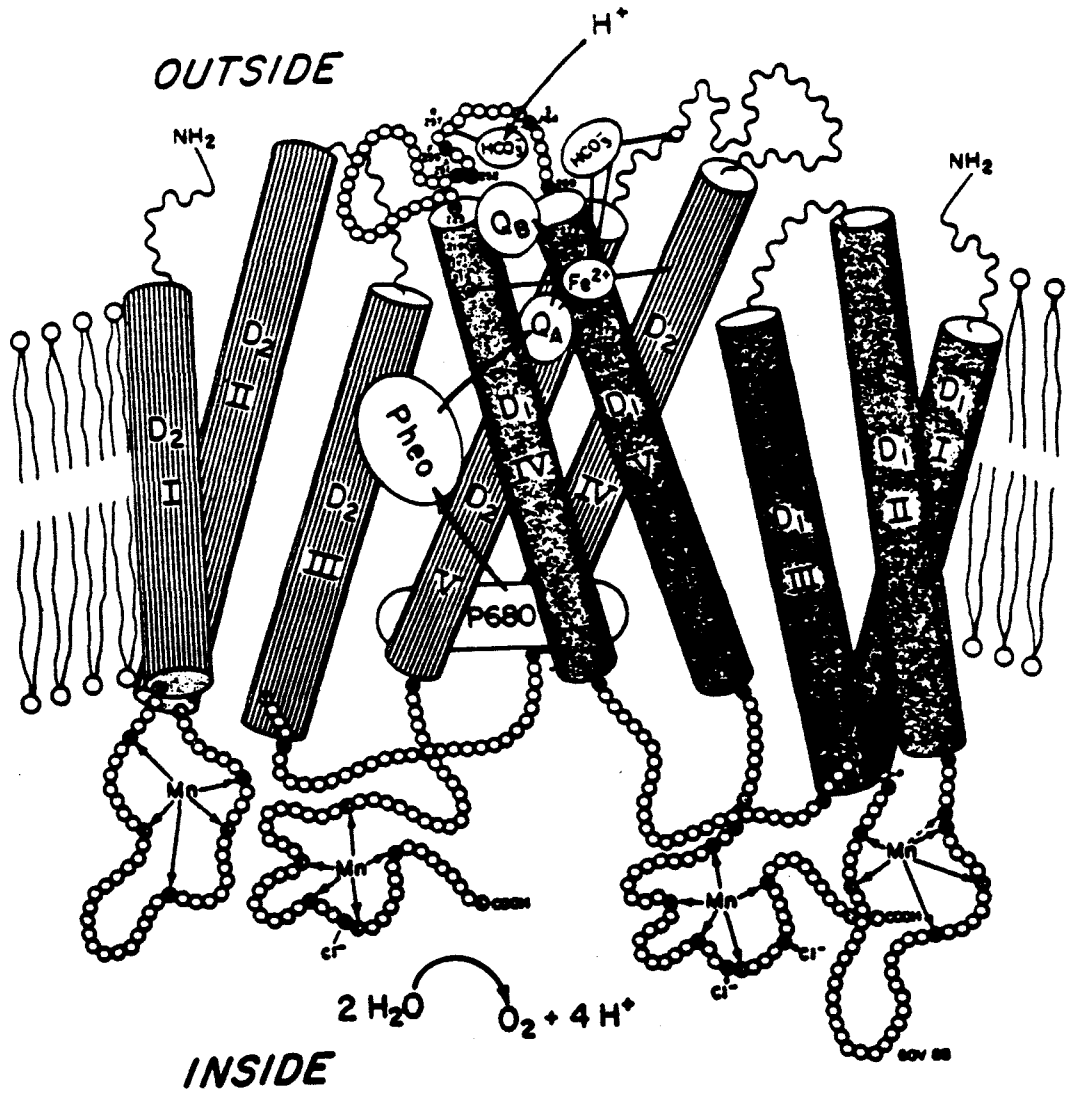


Fig. 3: *A highly-speculative working model of the reaction centre of photosystem II.* *(Contd.)*

Fig. 2: *A stylised model of the thylakoid membrane with four major protein complexes.*

The light-harvesting pigment-protein complexes have, however, been omitted. The depiction of photosystem II is adapted from Mathis (18) and the organisation of the cytochrome $b_{6/f}$ complex is based on that by Mansfield and Anderson (19) and by Ort (20). The organisation of PSI is adapted from Ort (20) and Malkin (21). The organisation of the H+ -ATPase (CF_1–CF_0) is highly schematic; each of the subunits in the membrane, perhaps, span the membrane. The hydrophobic CF_0 appears to contain 4–6 copies of the DCCD (N,N' dicyclohexylcarbodiimide) binding protein or subunit III but CF_0 has not yet been purified [see McCarty and Nalin (22)]. A model for isolated hydrophilic CF_1 (see the portion sticking out of the membrane) has been proposed by Tiedge *et al.* (23). The subunit stoichiometry shown here is $3\alpha\ 3\beta:1\gamma:1\delta:1\varepsilon$ (22). The symbols, used in the figure have the same meaning as in the legend of Fig. 1; Fe_2S_2 stands for Rieske iron sulphur centre. Molecular masses of the various polypeptides are denoted on the figure in kilodaltons (KD). Details of photosystem II, without the extrinsic polypeptides (17, 24 and 33 KD) and the 43 and 47 KD antenna proteins, and their associated symbols, are shown in Figure 3 and its legend. The figure is modified from that presented earlier by the author in (17); the modification is mainly in photosystem II and was first used by Eaton-Rye (24).

above. A similar, although not identical, effectiveness of Cl^- substitutes against heat damage [Coleman *et al.* (37)] and against dissociation of the extrinsic polypeptides [Homann (38)] has been observed. Hind *et al.* (4) suggested that the ionic volume may be the critical factor; that is, the smaller the anion the greater the activity. Perhaps a 'gate' or 'pocket' exists for the entry of the anion. Figure 5 shows that the Cl^- volume of 25Å is optimal for activity in spinach thylakoids. Critchley *et al.* (36) suggested that ionic volume is not the only factor because HCO_3^-, F^-, OH^-, and Acetate$^-$ that have smaller volume than Cl^+ gave low or no stimulation of electron flow (for function of HCO_3^- on electron acceptor side, see Ref. 17). A plot of the effectiveness of the anion as a function of ionic field (= $Q_A/e_S r_A^2$, where Q_A is the anion charge, r_A is the Pauling radius and e_S is the differential dielectric constant of water) showed that the effectiveness of anions decreases with decreasing ionic field. However, anions with large ionic field (HCO_3^-, SO_4^{2-}, Ac^-, PO_4^{3-}, OH^- and F^-) were also relatively ineffective in stimulating electron flow.

Coleman *et al.* (37) had suggested that the similarity of the specificity of the anions for stimulating electron flow and protection against mild heat damage is due to similar selective reversible binding of the anions to or near the O_2-evolving complex. Additionally, Homann (38; also see 39) has found that the anions also influence the binding of the 17 and 23 kDa extrinsic polypeptides to the membrane, implying a structural role of Cl^- proposed 36 years ago by Gorham and Clendenning (3). Any proposed mechanism of the Cl^- or substitute function must, therefore, include consideration of the anion volume, ionic field and hydration energy of the anion, and the ability to organise the structure (protein surface etc.)

IV. Binding Sites

Three major points are [see (15)]: (1) there are several (ranging from 4 to

Fig. 3 (*Contd.*)
D_1-I though V, equivalent to the L-subunit of the bacterial reaction centre, is the 32 kilodalton rapidly turning over herbicide-binding protein. D_2-I through V, equivalent to the M-subunit of the bacterial reaction centre, is the 34 kilodalton polypeptide. Approximate locations of the functional chlorophyll *a* P680, the functional pheophytin (Pheo), bound plastoquinone Q_A (on D_2), Fe^{2+}, and bound plastoquinone Q_B (on D_1) are shown. Q_A is *not* buried as shown, but is located there for the convenience of drawing. By analogy with photosynthetic bacteria, it is thought to be located at the same distance in the membrane as Q_B. The N-terminals of D_1 and D_2 face the outside, whereas, the C-terminals face the inside of the membrane. Suggested sites of HCO_3^- on Arginine-257, and on Fe^{2+} are also shown. Amino acids that are known to be involved in the herbicide niche are shown by solid dots; mutations at these sites are known to create herbicide resistance. Possible sites for Mn and Cl^- binding are suggested to be on the lumenal portions of the D_1 and D_2 polypeptides. In this model, H^+s are brought in via HCO_3^- from the outside and are released on the inner side when Cl^- is released. This composite model is based on concepts presented in references (15, 26, 27 and 28).

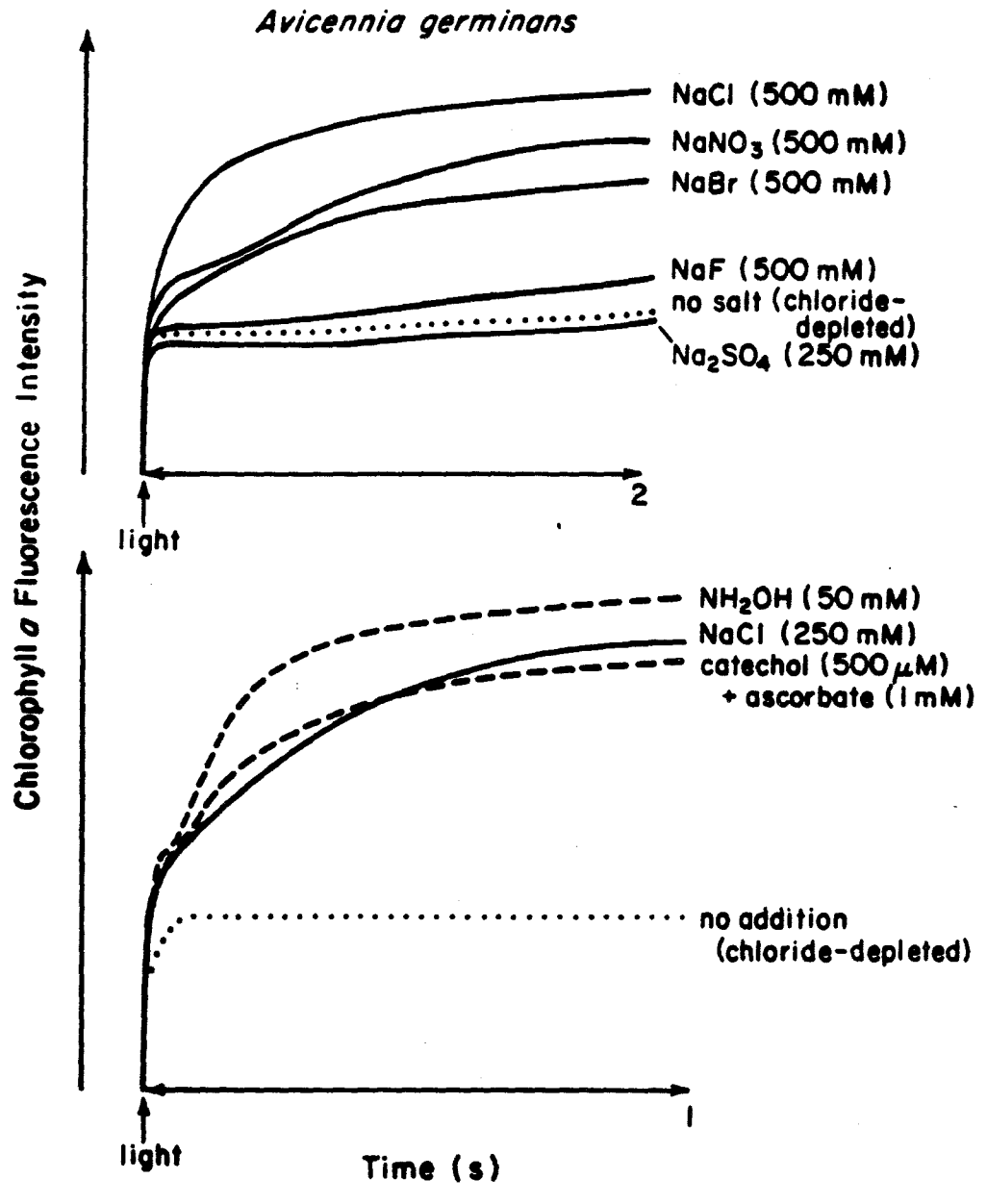


Fig. 4: *Chlorophyll a fluorescence transients in halophyte thylakoids.*

Top curves: in the absence of added salt (no salt) and in the presence of NaCl, $NaNO_3$, NaBr, NaF, and Na_2SO_4. *Bottom curves*: in the absence of added chloride ('no addition'), in the presence of NaCl ('NaCl') and in the presence, without added chloride, of electron donors to PSII: NH_2OH and reduced catechol. [After Critchley *et al.* (36).]

40) binding sites; (2) there are two types of binding sites: (a) catalytic sites on D_1 and D_2 polypeptides; and (b) regulatory on 33 kDa extrinsic polypeptide; and (3) removal of extrinsic polypeptides (e.g., 17 and 23 kDa) increases the $[Cl^-]$ requirement by ten times—further removal of 33 kDa increases, by another ten fold, the $[Cl^-]$ requirement.

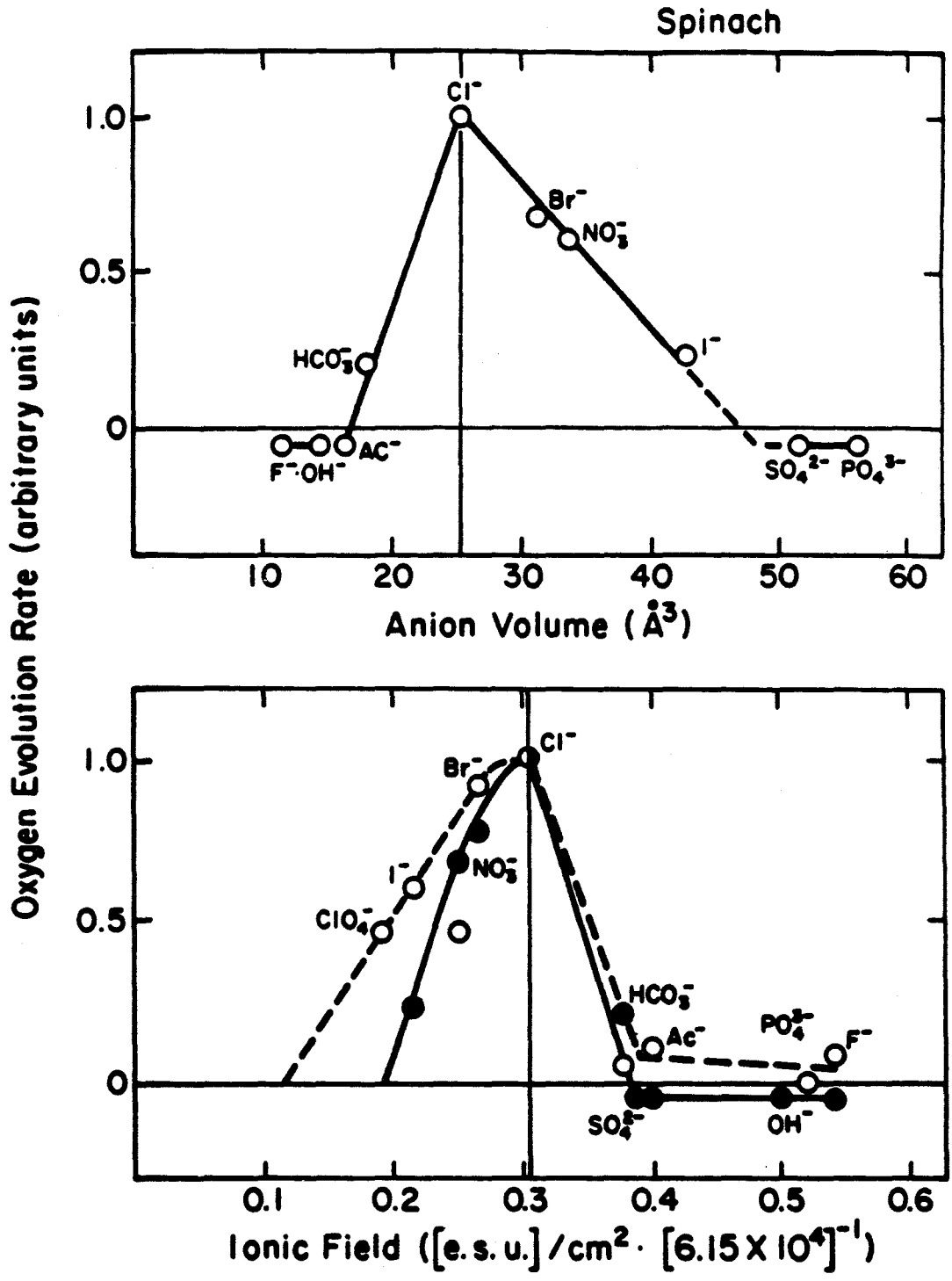


Fig. 5: *Hill reaction rates.*

Rate of O_2 evolution, with ferricyanide as electron acceptor, versus the anion volume and ionic field for spinach thylakoids. Data from Kelley and Izawa (6). [After Critchley *et al.* (36).]

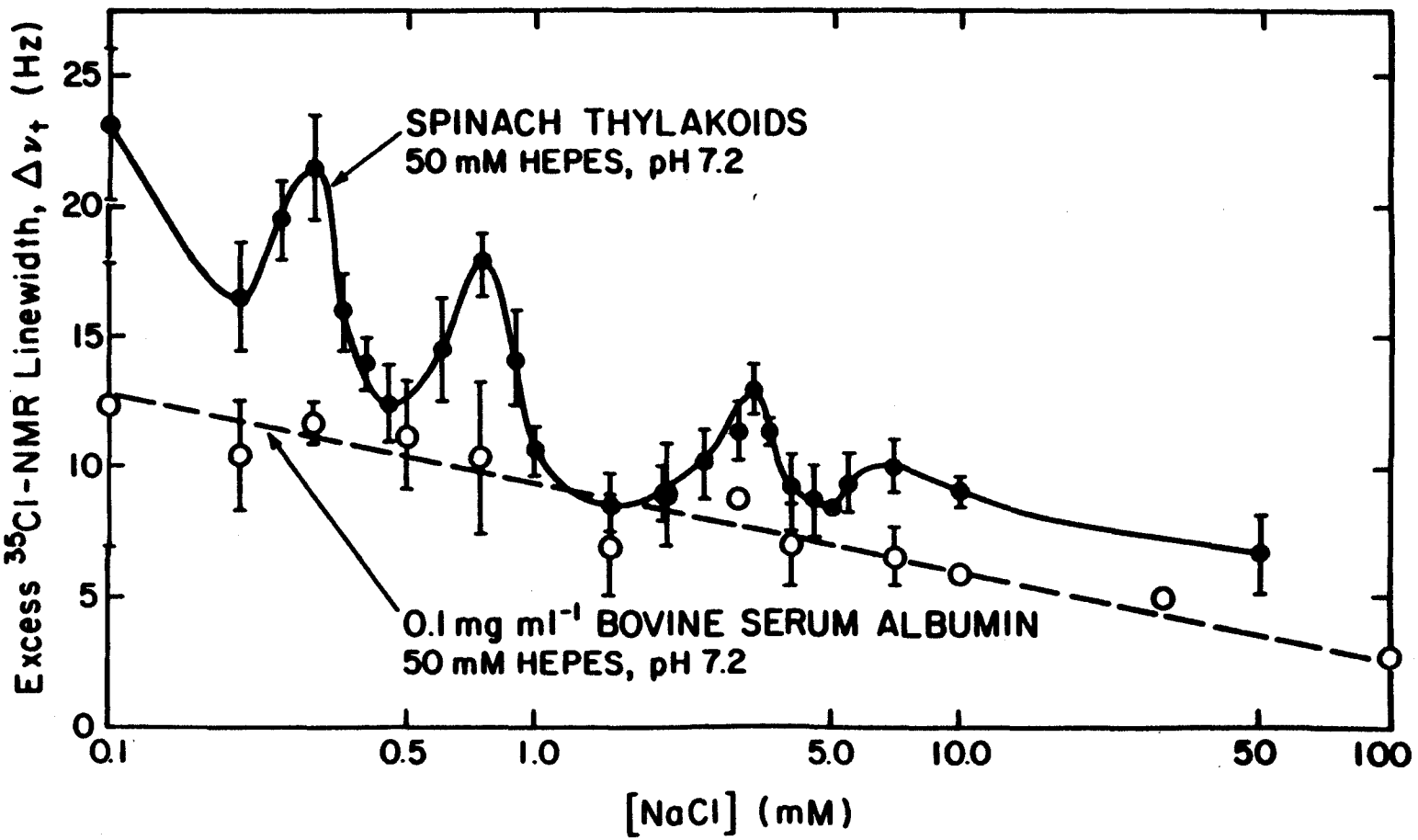
Number of Binding Sites. Using radioactive ^{36}Cl, Theg and Homann (40) obtained a value of 10 Cl^-/600 Chl in spinach thylakoids. Izawa (41) obtained a value that ranged from 4 to 40 Cl^-/600 Chl. Using ^{35}Cl NMR results, Baianu *et al.* (42) and Govindjee *et al.* (10) estimated a value of 20–40 Cl^-/600 Chl in thylakoids from halophytes, suggesting that multiple binding sites exist for Cl^-.

Multiple Binding Sites. The existence of multiple sites of Cl^- was most dramatically demonstrated by ^{35}Cl-NMR measurements on photosystem II and thylakoid membranes from spinach [see Coleman *et al.* (43–46); Fig. 6]. Plots of ^{35}Cl NMR linewidth, corrected for that in chloride solution, show several maxima that occur at roughly the same [Cl^-] as intermediary plateaus in Hill activity (at low light intensities) versus [Cl^-] plot, indicating a possible correlation between the two measurements (44).

These maxima were obtained from data collected by an NMR instrument at the University of Illinois at Urbana (NSF-250; 250 MHZ homebuilt NMR spectrometer; spectra obtained at 24,508 KHz using a 33μs 90° pulse and 360 ms recycle time; signals detected in quadrature with 32K data points and a spectral width of ± 25,000 Hz; signals were then block averaged (500 scans per block) and transferred to a Nicolet 1180E computer [for further details, see Ref. (44)].

No such maxima were obtained in the NMR linewidths of (a) Cl^-/buffer solutions containing 0.1, 1.0 and 10mM Cl^-; these linewidths were found to have exactly the same values (differences of Hz or less); thus, there was no apparent concentration dependence of the ^{35} Cl-NMR linewidth in the absence of proteins; and (b) highly purified bovine serum albumin (a Cl^- binding protein) in the same [Cl^-] range as used for PSII membranes [see Ref. (44)].

Furthermore, (1) NMR linewidths for PSII at the 'maxima' depended directly on the activity of the preparations (44), suggesting that whatever structures contribute to the linewidth maxima also contribute to the overall activity; (2) each maximum appears to have a different degree of sensitivity to the inactivation of the Hill reaction (43–46), suggesting that each maximum reflects a discrete structural entity; (3) mild heating (46); Tris (to remove Mn and 18,24 and 33kDa polypeptides), salt-washing (to remove 18 and 24 kDa; and 33 kDa polypeptides) and hydroxylamine (to remove Mn) (45) treatments show different effects on the different NMR linewidth maxima; (4) the NMR linewidth maxima are altered by removing 18 and 24 kDa polypeptides by NaCl washing (as mentioned above) but 3 of the 4 maxima were restored by adding 2mM $CaSO_4$; this also demonstrates another correlation with Hill activity, which is also partially restored by adding Ca^{2+}; (5) ^{35}Cl-NMR line-bradening in the presence of PSII membranes in the [Cl^-] range between 0.1 mM and 0.3 mM was eliminated by a very low concentration of added Br^- (0.1 mM); the observed reduction in the height of the first linewidth maximum is also consistent with the competition between the anions for a limited set of binding sites.


Excess ^{35}Cl-NMR Linewidth, $\Delta\nu_+$ (Hz)
25
20
15
10
5
0
SPINACH THYLAKOIDS
50 mM HEPES, pH 7.2
0.1 mg ml^{-1} BOVINE SERUM ALBUMIN
50 mM HEPES, pH 7.2
0.1
0.5
1.0
5.0
10.0
50
100
[NaCl] (mM)

Two Major Binding Domains. Since several of the ^{35}Cl-NMR maxima, discussed above, occur at higher [Cl^-] than that needed for activating O_2 evolution, it is suggested that these may be related to the *regulatory*, rather than the catalytic, function of Cl^-. Mild heating (3 min. 30°C) eliminates the high [Cl^-] 'kinks' of maxima and minima in both the [Cl^-] dependence of the $^{35}Cl^-$NMR linewidth and the Hill reaction (46). Furthermore, these are the ones that are dramatically altered by the removal of the regulatory proteins (18 and 24 kDa) and partially restored by the addition of Ca^{2+} (45).

The [Cl^-] dependence of excess ^{35}Cl-NMR linewidth data in halophytes [Baianu *et al.* (42)] and in spinach (44–46) show a general pattern: there is a large excess linewidth at low [Cl^-] that decreases with increasing [Cl^-]. Such a curve suggests that the tightly binding sites are filled at low [Cl^-]. We suggest that this tightly binding site at the lowest [Cl^-] is related to the catalytic function. However, Cl^- affinity at this site decreases when the 33kDa extrinsic polypeptide is also removed: a peak appears in the [Cl^-] dependence of ^{35}Cl-NMR linewidth at ~ 0.5 mM. This is qualitatively consistent with the idea that the [Cl^-] requirement for the O_2 evolution activity of such preparations is extremely high. Further research is necessary to correlate ^{35}Cl binding with the O_2 evolution since binding is required for O_2 evolution, but binding is not a sufficient condition for O_2 evolution. It can be easily seen that removal of Mn by NH_2OH, that eliminates O_2 evolution, only slightly alters the [Cl^-] dependence of the ^{35}Cl-NMR linewidth (45).

V. Mechanism of Cl^- Action

We have already mentioned (see section II above) that Cl^- functions on the electron donor side of PSII between or at the charge accumulator M and 'Z' where several artificial electron donors donate electrons. Now, the question is: What is its mechanism of action? First of all 'M' exists in several redox states, labelled as S-states [see (29-31) and Wydrzynski (47)].

Fig. 6: *^{35}Cl-NMR binding curve for thylakoid membranes and for bovine serum albumin (BSA).*

The buffer was 50 mM HEPES at pH 7.2; the chlorophyll concentration wa 0.5 mg. ml^{-1}. BSA was dissolved at a concentration of 0.1 mg. ml^{-1} in 50 mM HEPES at pH 7.2. $\Delta\nu_t$ (Hz) = excess line width; it is equal to $\Delta\nu_{obs}$ (observed linewidth at half-maximum intensity) minus $\Delta\nu f$ (linewidth at half-maximum intensity for Cl^- solution). $\Delta\nu_{obs} = \Delta\nu_{bound}\, f_{bound} + \Delta\nu f\,(1\text{-}f_{bound})$, where $\Delta\nu_{bound}$ is the weighted average of the contributions from Cl^- in the bound state, $\Delta\nu f$ is the same in the free state, and f_{bound} is the fraction bound. Since $\Delta\nu_b$ (approximately 10kHz) is very much larger than $\Delta\nu_f$ (approximately 12–30 Hz, depending on the viscosity) and $f_{bound} << 1$ (for a dilute protein solution), $\Delta\nu_t = \Delta\nu_{bound}\, f_{bound}$. The details of the NMR instrument and its settings are given in the text. [After W. J. Coleman, unpublished; also see ref. (44).]

In darkness, 'M' is in state 'S_1', and the following sequence takes place in the first cycle in a dark-adapted sample:

$$S_1 \xrightarrow{h\nu_1} S_2 \xrightarrow{h\nu_2} S_3 \xrightarrow{h\nu_3} S_4 \longrightarrow S_0 \quad (2H_2O \rightarrow O_2)$$

And, the following sequence occurs in the subsequent cycle(s):

$$S_0 \xrightarrow{h\nu_4} S_1 \xrightarrow{h\nu_5} S_2 \xrightarrow{h\nu_6} S_3 \xrightarrow{h\nu_7} S_4 \longrightarrow S_0 \quad (2H_2O \rightarrow O_2)$$

Here, $h\nu_n$ stands for a light flash, and S_n for a redox state of 'M', the higher n's representing higher oxidation states. Thermoluminescence [see Sane and Rutherford (48)] is a powerful tool to monitor the S-states because it represents the back reaction of PSII involving the S-states and the electron acceptor side. Thermoluminescence is suggested to appers as follows:

$$S_2 \cdot Z \cdot P680 \cdot Pheo \cdot Q_A \cdot Q_B^- \text{ (created by light reaction)} \xrightarrow{\text{warm}}$$

$$S_1 \cdot Z^+ \cdot P680 \cdot Pheo \cdot Q_A^- \cdot Q_B \xrightarrow{\text{warm}} S_1Z \cdot P680^+ \cdot Pheo^- \; Q_A \; Q_B \xrightarrow{\text{warm}}$$

$$S_1 \cdot Z \cdot P680^* \cdot Pheo \cdot Q_A \cdot Q_B \xrightarrow{\text{spontaneous}} S_1 \cdot Z \cdot P680 \cdot Pheo \cdot Q_A \cdot Q_B + \text{light}$$

This light appears as a glow peak 'B' in thermoluminescence curves and is said to originate from an S_2Q_B back reaction. On the basis of their thermoluminescence results, Homann *et al.* (49) and Vass *et al.* (50) suggested that Cl^- depletion generates an abnormal 'S_2' state. It was, however, Muallem *et al.* (7) who had first suggested that Cl^- depletion causes the formation of defective oxidant storage. Several investigators, using a variety of experimental approaches, have confirmed this conclusion [see *e.g.* (50–53)]. Flash illumination of dark-adapted Cl^- depleted preparations generates an abnormal S_2-state (labelled as 'Σ_2 state') which has an abnormally long lifetime and has a lower than normal oxidation potential [Homann *et al.* (49)]. This Σ_2 state, unlike the normal S_2 state, does not produce the normal multiline ESR signal for manganese [see Damodar *et al.* (54) and Imaoka *et al.*(55)]. However, the g = 4.1 signal of the S_2 state is still present in Cl^- depleted samples [Ono *et al.* (56)]. Addition of Cl^- restores the normal S_2 signal. ^{35}Cl-NMR linewidth broadening, suggestive of Cl-binding, appears to be dependent upon the S-state transitions. Preston and Pace (57) showed its relationship to the $S_2 + S_3$ states.

In order to formulate a theory for the mechanism of Cl^- action, it is useful to keep in mind the following points [Homann (13); Coleman and

Govindjee (58)]: (1) Inactivation by Cl^- depletion is reversible; it appears that only electrostatic interactions are involved; (2) Cl^- depletion is accelerated by incubating the thylakoids at high pH; (3) Cl^- binding is pH dependent and reversible; (4) activation of the Hill reaction by added Cl^- shows hyperbolic kinetics, indicating saturation; (5) activation of the Hill reaction by anions is relatively, but not exclusively, specific for Cl^-, the selectivity does not resemble the lyotropic Hofmeister series; and there is a competitive interaction of anions; (6) the pH optimum of the Hill reaction is shifted to more alkaline pH by Cl^- binding; this suggests that Cl^- addition is equivalent to an acidification; Cl^- may be involved in splitting of H_2O and protonation steps; and (7) an interaction of Ca^{2+} with the Cl^- effect exists.

To this check list, we may add [also see Homann (13)] the following unique properties of Cl^- in the water oxidising steps: (1) there are two types of Cl^- binding/interactions (see Section IV); (2) Cl^- binding is controlled by a group with rather low pKa; (3) there is modulation of Cl^- binding by the extrinsic polypeptides [see Section IV; also see Homann (59)]; (4) prevention of binding of other anions and chemicals by Cl^-; (5) effect of sulphate ions on Cl^- action suggesting that its interaction involves deprotonation, and (6) the variation of Cl^- binding during S-state transitions [see (57)].

There are two major effects of Cl^-: a regulatory (a structural) role; and a catalytic role. We shall discuss here only the latter. Govindjee *et al.* (10) suggested that the function of Cl^- was to act as a counterion to positive charges on the oxygen-evolving complex. Since these charges arrive after each light flash, it was suggested that Cl^- binding occurred after each S-state transition. The release of Cl^- was associated with the release of a positive charge, i.e. a proton (H^+). Since there is no proton release during $S_1 \rightarrow S_2$ transition, the unbinding of Cl^- was suggested not to occur after this transition. Thus, the absence of Cl^- could specifically affect the S_2 state created. In this hypothesis, the function of Cl^- was to bind to the system when a positive charge arrived on the water oxidation complex, stabilising the system, and to lead the H^+s away from the system by simultaneous unbinding from the system. The reversible binding of Cl^- (high exchange rate) and the low binding energy (~ 3Kcal) [see Baianu *et al.* (42)] supported this picture.

Coleman and Govindjee (58) proposed a detailed picture as to how Cl^- activates the base catalysis, i.e. how it activates the H^+ removal from water (see Fig. 7). Here, a proton accepting group B^- functions to extract H^+s from H_2O, whereas Mn functions to extract an electron from H_2O, and Cl^- functions to bind transiently (and thus, reversibly) to a positively charged group N^+ that raises the pKa of B^-. The N^+ may represent an amino acid histidine (see Fig. 3), and B^- may represent a proton accepting group (Fig. 3). Thus, as Cl^- binds to N^+, the pKa of the nearby B^- changes, and this increases it affinity to catalytic H_2O protons at the

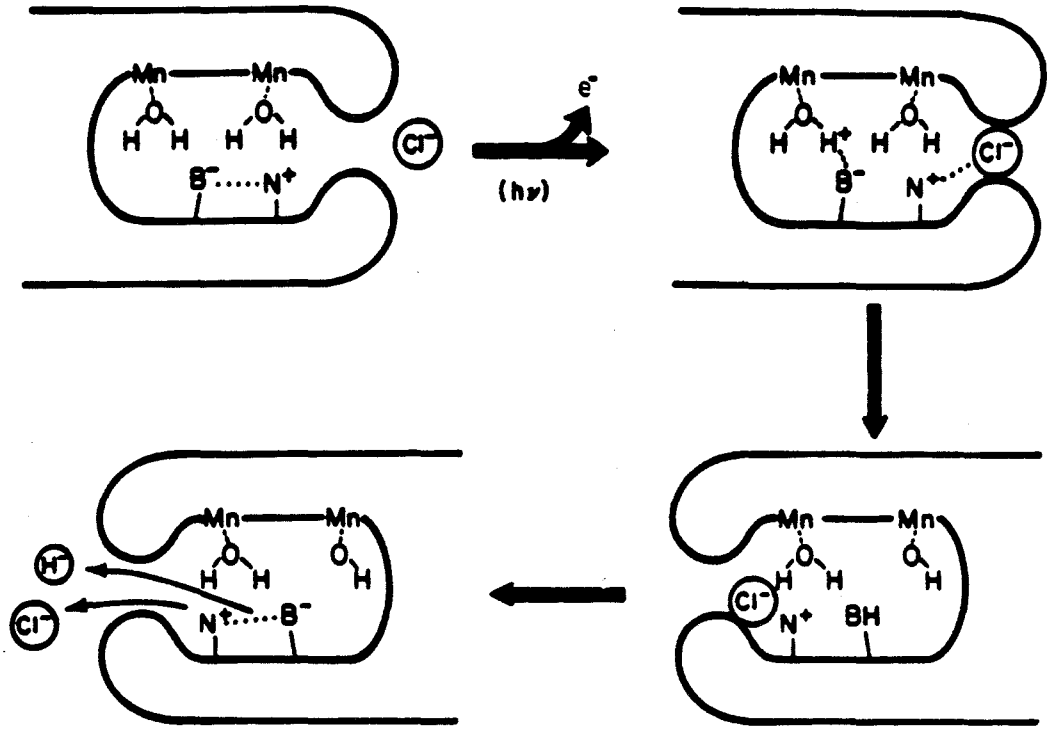


Fig. 7: *A working model for Cl^- activation of O_2 evolution.*
Here binding of the anion to a basic amino acid (N^+) shifts the pKa of a reactive group (B^-; an acidic amino acid) on the lumenal side of D_1 and D_2 polypeptides (also see Fig. 2). This makes B^- more reactive in 'pulling' water protons from water molecules bound at the active site. As soon as BH is made, H^+ (bound at B^-) and Cl^- (on N^+) leave the binding sites restoring the system to its original state. In this hypothesis, Cl^- is reversibly bound and unbound. [Modified after Coleman and Govindjee (58).]

Mn-site making it easier to 'pull' H^+s out of water, as 'Mn' pulls electrons from H_2O. Both the H^+s and Cl^-s leave the catalytic site at the same time. H^+s may then be conducted through the 33 kDa Cl^- -binding extrinsic polypeptide to the outside, i.e., into the lumen (for details, see [15]). For yet another, but somewhat similar picture, see Homann (13).

The pH profile of the anion requirement, and the correlation of the anion volume and the hydration energies of the activating anions with their effectiveness, suggest [see Govindjee and Homann (60)] that protonation of unique functional groups are critical for the mechanism of water oxidation. This supports the belief of Homann (13) [also see Coleman and Govindjee (15, 58)] that one function of the anions is to organise the protein surfaces and suitable H^+ acceptor/donor groups at the active site of the water oxidase, thereby facilating the proton abstraction from substrate water during its oxidation to molecular O_2. Further research is needed before we can understand the molecular mechanism of Cl^- action; only a modest beginning has been made thus far.

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