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APPLICATIONS OF ^{35C}L-NMR TO THE STUDY OF CHLORIDE BINDING IN THE OXYGEN EVOLVING COMPLEX OF PHOTOSYSTEM II

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ABSTRACT

^{35}Cl-NMR has become a powerful new tool for studying Cl^- activation of photosynthetic oxygen evolution. This approach opens up the possibility of directly monitoring Cl^- binding within the enzyme. Here we discuss both the pioneering application of this technique to thylakoids from salt-tolerant plants, as well as the latest discoveries regarding Cl^- binding to spinach Photosystem II. Knowledge of the binding mechanism gained from these investigations will make it possible to construct a detailed model for the role of Cl^- in oxygen evolution.

INTRODUCTION

It has been known for many years that chloride ion (Cl^-) is required for photosynthetic oxygen evolution in isolated chloroplasts, but its mechanism of action still remains a mystery. Although numerous detailed kinetic studies of the "Cl^-effect" have been reported (see review {1,2}) the information gained by this kind of approach is not, by itself, sufficient to construct a model for Cl activation of Photosystem II (see Fig.1). In all enzyme investigations, the independent measurement of the binding properties of an activating ligand is crucial in determining the role of the ligand in the activation process, since this data may indicate, among other things, the true affinity of the enzyme for the activator, and the location of the binding site or sites.

^{35}Cl-NMR has been increasingly used to examine the binding of Cl to various proteins (see ref. {3} for hemoglobin, for example). Chloride is not a very sensitive NMR nucleus, however; for this reason, the first NMR observations of Cl binding to the oxygen-evolving complex (OEC) of Photosystem II (PSII) were performed on thylakoid membranes isolated from extremely salt-tolerant plants (halophytes), which posses a high Cl requirement for oxygen evolution (5,6). More recently, this approach has been extended to spinach thylakoids and PS II particles (7). In the case of spinach thylakoids, the apparent dissociation constant for Cl (K_A) is only 0.6-0.9 mM (4,5) and ^{35}Cl-NMR binding studies on them have only now become feasible. This was made possible because of advances in instrumentation.

THEORY

When measured by ^{35}Cl-NMR, an aqueous solution of sodium chloride gives a relatively narrow Lorentzian line with a full width at half-height of about 14 Hz. The line is considerably broadened, however, in the presence of thylakoid membranes, which contain Cl binding proteins (see Fig.2). This broadening was shown in (6) to result primarily quadrupolar interactions between the ^{35}Cl nuclei and the fluctuating electric field gradients at the membrane surface.

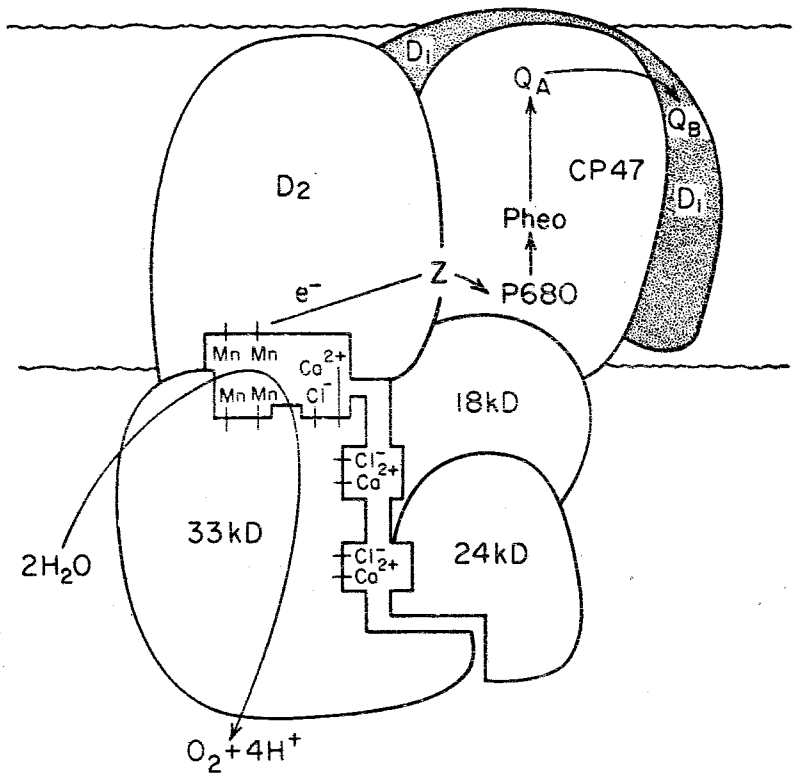

Figure 1. A working model of Photosystem II and the oxygen evolving complex (OEC) in spinach thylakoids. The proteins known as CP 47, D_1, and D_2 are suggested here to comprise a triad which forms the heart of the reaction center. It is possible that some of the core chromophores may share ligands from more than one protein. "P680" denotes the primary reaction centre chlorophyll a; "Pheo" is pheophytin, the primary electron acceptor; "Q_A" is a bound plastoquinone, the first stable electron acceptor; "Q_B" is a bound plastoquinone which transfers electrons to other components in the chain "Z" is an electrons from the OEC. The OEC is assumed here to include D_2, along with polypeptides of molecular weight 18, 24 and 33 kD. The manganese involved in water splitting has been suggested to reside on both D_2 and the 33kD polypeptide. We suggest here that the latter polypeptide probably also possesses the primary binding sites for Cl^- and Ca^{2+}, but the 18 and 24 kD proteins are required for high affinify binding of Cl- and Ca^{2+} *in vivo*.

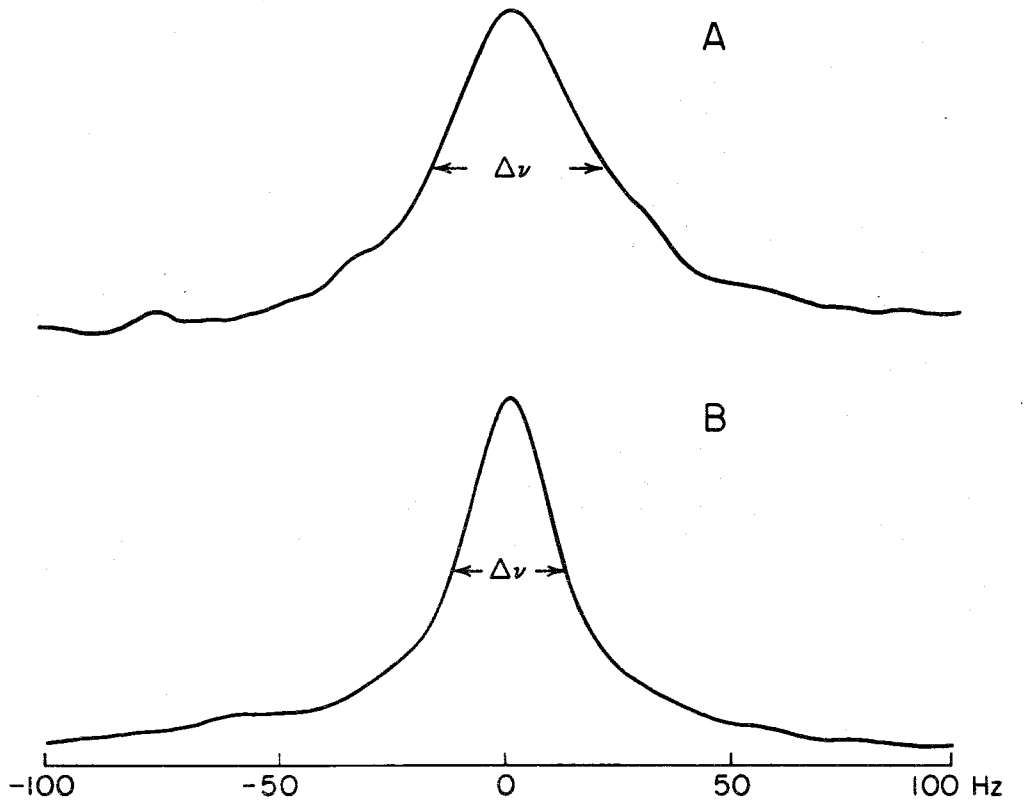

Fig. 2. ^{35}Cl-NMR line broadening in the presence of spinach thylakoids. (A) Spectrum for thylakoids in 0.75 mM NaCl at pH 7.2 and 1.0 mg Chl per ml. (B) 10mM MaCl in Buffer. Spectra were obtained at 24.51 MHz, using a 20 mm sideways-spinning cell. Final linewidths include 10Hz of exponential line-broadening.

A ^{35}Cl ion bound to the thylakoid is predicted to have a Lorentzian lineshape, and contributes a linewidth (8) given by :

$$\Delta \upsilon_b = (2/5)\,\pi\,(e^2 qQ/h)^2\, \tau_c \qquad .....1$$

where e is the electronic charge, eq is the electric field gradient experienced by the nucleus, eQ is the electric quadrupole moment of the nucleus, and τ_c is the correlation time for fluctuations in the electric field gradient.

For the case of rapid exchange of Cl^- between bound and free environments (6), the observed NMR linewidth at half-height Cl^- is the weighted average of the contributions from Cl^- in the bound state and the free state :

$$\Delta\, \upsilon_{obs} = \Delta\, \upsilon_B\, f_B = \Delta\, \upsilon_f\, (1-f_B) \qquad .....2$$

The contribution from each site is proportional to the fraction of Cl^- bound (f_B).

It is more convenient, however, to plot the net or excess linewidth, which reflects the amount of Cl^- actually bound. Thus, for $\Delta\upsilon_b >> \upsilon_f$ and fB << 1:

$$A\nu_t = \Delta\upsilon_{obs} - \Delta\upsilon_f \cong \Delta\upsilon_B\, f_B \qquad .....3$$

For a simple system, eqn. 3 would be expected to produce a binding curve for υ_t vs. (Cl^-) in the form of a descending hyperbola, since f_B decreases with increasing (Cl^-).

NMR ESTIMATES OF K_A

The type of binding curve described above has been obtained for thylakoids isolated from the halophyte *Avicennia germinans* (Fig.3; see ref. {6}). These data were used to calculate the dissociation constant for Cl^-, since K_A ultimately

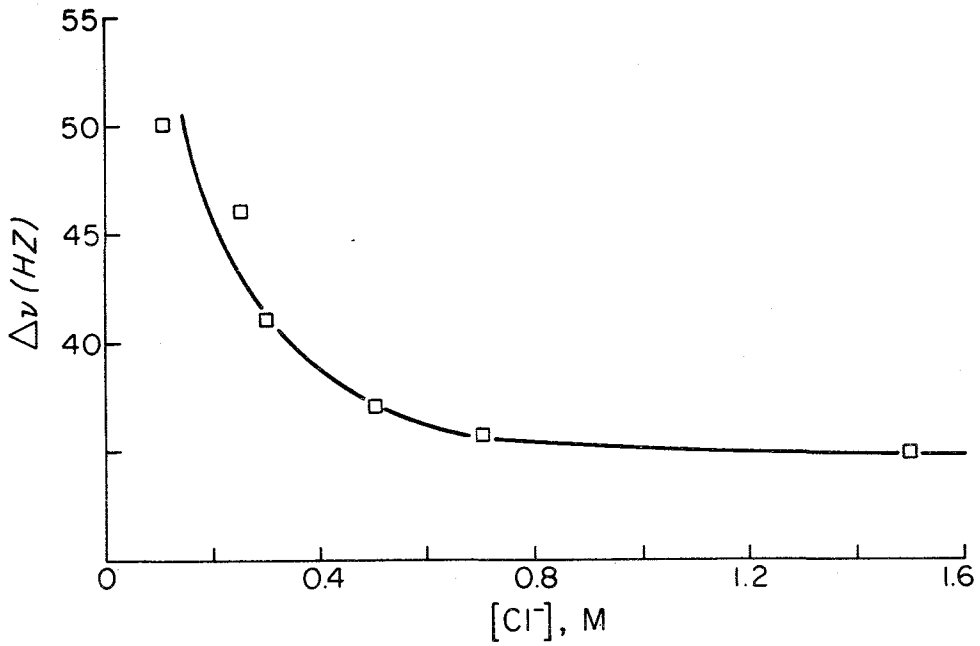

Fig. 3. Dependence upon [Cl-] of ^{35}Cl line broadening by thylakoids of *Avi*, germinans at pH 7.2, 25°C, 2.9 mg of Chl per ml. Redrawn from ref. 6.

depends on f_B (6). Values obtained for K_A were 0.1M and 0.14 M at 5°C and 25°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 Kcal mol^{-1} for Cl^- in this system, which indicates weak ionic binding (6).

Unfortunately, calculation of the apparent dissociation constant from O_2 evolution activity measurements in the same halophyte thylakoids gives a value ($KA \leq 0.014M$) which is an order of magnitude lower than the K_A values obtained from the NMR measurements. This discrepancy may be due to the fact that the NMR measurements were performed at Cl^- concentrations (0.1-2.0M Cl^-) that were still very much greater than K_A. Nevertheless, these early experiments demonstrated the feasibility of using $^{35}Cl^-$-NMR to measure the binding of Cl^- in isolated thylakoids.

THE pH DEPENDENCE OF Cl - BINDING

Theg and Homann (9) have demonstrated that in spinach thylakoids the binding of Cl^- is apparently coupled to the binding of protons. Critchley *et al.* (5) therefore measured the pH dependence of both oxygen evolution and Cl^-binding in halophytes, in order to determine the nature of the binding mechanism and its relationship to enzyme activity. They found, among other things, that Cl^- binding to thylakoids from halophytes (as monitored by $^{35}Cl^-$-NMR) is strongly pH dependent, and that the bellshaped pH-dependence of the binding roughly parallels the pH-dependence of oxygen evolution (Fig.4; see ref. {5}).

In addition, this pH-dependent binding was eliminated by mild heating, a treatment which is known to specifically inhibit oxygen evolution (10). Critchley *et al* .(5) explained their NMR

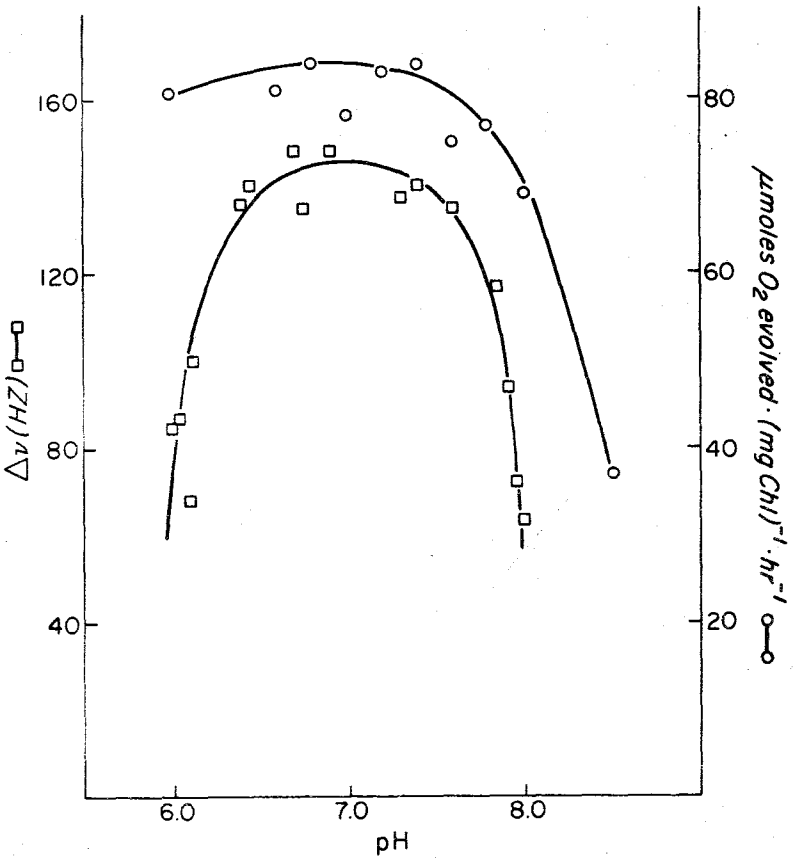

Fig. 4. The pH dependence of O_2 evolution with ferricyanide as electron acceptor (open circles) and ^{35}Cl-NMR linewidths (open squares) in thylakoids from Avi. germinans, isolated at pH 7.6, with 6 mg Chl/ml and 1M NaCl. Redrawn from ref. 5.

data by proposing that Cl^- activates the OEC by reversible, pH dependent ionic binding. This idea has now become a key element of some models for Cl^- stimulation of oxygen evolution (11,12,13).

EFFECTS OF FLASH ILLUMINATION ON CL⁻ BINDING

Preston and Pace (14) have recently studied the effect of single-turnover flashes of light on Cl^- binding in mangrove (*Avicennia marina*) PS II particles by $^{35}Cl^-$ NMR. They report that the affinity of the enzyme for Cl^- is much greater in the S_2 and S_3 (light-induced, oxidized states of the OEC; see ref. 15 for a discussion of the S-states) than in the S_1 (dark) state. Preston and Pace also found that the net amount of Cl^- binding (S_N-S_1) oscillated with a period of 4, which suggests that this binding may be coupled to the sequential turnover of the S-states of the OEC.

^{35}C I -NMR MEASUREMENTS OF SPINACH THYLAKOIDS AND PS II PARTICLES

Recently, it has become possible to obtain $^{35}Cl^-$NMR binding curves for spinach preparations, which have a very low dissociation

constant for Cl. Coleman *et al.* (7) have found that the Cl^-NMR binding curve for spinach thylakoids and PS II particles correlates with the activity curve for Cl^- activation of the Hill reaction (H_2O-2,6-Dichlorophenolindophenol.). The binding curve is interrupted, however, by sharp increases in linewidth at certain specific Cl^- concentrations. This behaviour may reflect cooperativity in the binding of Cl^-. The observed Cl^- binding is sensitive to both heating and Tris, treatments which inhibit oxygen evolution. [35] Cl^-NMR binding curves have also been obtained for spinach PS II particles (W. Coleman, *et al.*). This system has the advantage of allowing selective removal of the extrinsic OEC polypetides by salt washing. The PS II Cl^- particles yield a Cl^- binding curve very similar to that for thylakoids, although the linewidth maximum for the highest $[Cl^-]$ is greatly reduced. Removal of the 18 and 24 KD polypetides has two effects : (1) the overall linewidth is greatly reduced (indicating reduced Cl^- affinity) and (2) the sharp increases in linewidth (maxima) are replaced by sharp decreases in linewidth (minima) at the same Cl^- concentrations. The "normal" Cl^- binding curve can be completely restored, however, simply by adding 2.0 mM $CaSO_4$, to the medium, even if the 18 and 24 KD proteins are absent. This result indicates that Ca^{2+} is vitally important for the high-affinity binding of Cl^-, since oxygen evolution is completely inhibited in the absence of both of these effectors.

Further studies of Cl^- binding in these high affinity systems, along with manipulation of the OEC polypeptides, will be needed to locate the Cl^- binding sites and to determine their role in oxygen evolution.

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