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^{35}Cl-NMR measurement of chloride binding to the oxygen-evolving complex of spinach Photosystem II

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The mechanism by which Cl^- activates the oxygen-evolving complex (OEC) of Photosystem II (PS II) in spinach was studied by ^{35}Cl-NMR spectroscopy and steady-state measurements of oxygen evolution. Measurements of the excess ^{35}Cl-NMR linewidth in dark-adapted, Cl^--depleted thylakoid and Photosystem II membranes show an overall hyperbolic decrease which is interrupted by sharp increases in linewidth (linewidth maxima) at approx. 0.3 mM, 0.75 mM, 3.25 mM (2.0 mM in PS II membranes), and 7.0 mM Cl^-. The rate of the Hill reaction ($H_2O \rightarrow$ 2,6-dichlorophenolindophenol) at low light intensities (5% of saturation) as a function of $[Cl^-]$ in thylakoids shows three intermediary plateaus in the concentration range between 0.1 and 10 mM Cl^- indicating kinetic cooperativity with respect to Cl^-. The presence of linewidth maxima in the ^{35}Cl-NMR binding curve indicates that Cl^- addition exposes four types of Cl^- binding site that were previously inaccessible to exchange with Cl^- in the bulk solution. These results are best explained by proposing that Cl^- binds to four sequestered (salt-bridged) domains within the oxygen-evolving complex. Binding of Cl^- is facilitated by the presence of H^+ and vice versa. The pH dependence of the excess ^{35}Cl-NMR linewidth at 0.75 mM Cl^- shows that Cl^- binding has a maximum at pH 6.0 and two smaller maxima at pH 5.4 and 6.5 which may suggest that as many as three groups (perhaps histidine) with pK_a values in the region may control the binding.

Introduction

The oxygen-evolving complex of Photosystem II in green plants requires chloride in order to catalyze the light-driven oxidation of water with high efficiency [1–4]. The requirement for Cl^- is not exclusive, since other monoanions also activate the enzyme. Their effectiveness follows the order $Cl^- > Br^- \gg NO_3^- > I^-$ [1,3,4]. Although many details are known about the kinetics of Cl^- activation of O_2 evolution (for reviews, see Refs. 5–7), the information gained through this kind of study is not, by itself, sufficient to construct a model for the mechanism of Cl^- activation. In a system as

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Abbreviations: PS II, Photosystem II; OEC, oxygen-evolving complex; BTP, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; DCBQ, dichlorobenzoquinone; DCIP, 2,6-dichlorophenolindophenol; DPC, 1,5-diphenylcarbazide, PMA, polymethacrylic acid; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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structurally intricate as the OEC, it is important to study the Cl^- binding more directly and to address questions about the number of Cl^- binding sites and their location within PS II.

^{35}Cl-NMR has been widely used to examine the binding of Cl^- to various proteins [8–11]. Chloride is not a very sensitive NMR nucleus, however. For this reason, the first NMR observations of Cl^- binding to thylakoid membranes were performed on preparations from the leaves of extremely salt-tolerant plants (halophytes), since they have a high Cl^- requirement for O_2 evolution [4,12]. In the case of thylakoids from spinach, the apparent activator dissociation constant for Cl^- (K'_A) is only 0.6–0.9 mM [3,4], and thus ^{35}Cl-NMR binding studies on them have only recently been made feasible by advances in instrumentation. We therefore present here the first direct measurements of Cl^- binding to Cl^- depleted spinach thylakoids and PS II membranes in the Cl^- oncentration range (approx. 0.3 mM) where O_2 evolution is most sensitive to the activator.

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 Cl^--depleted samples. Thylakoids were prepared by mixing washed spinach leaves (chopped and deveined) in ice-cold grinding buffer consisting of 2 mM ethylenediaminetetraacetic acid (EDTA), 0.1% bovine serum albumin, and 50 mM sodium phosphate or hydroxyethylpiperazineethanesulfonic acid (pH 7.2) at a proportion of 2 ml buffer per g leaves. After grinding for 8 s at high speed in a Waring blendor, the homogenate was filtered through 12 layers of cheesecloth into an equal volume of chilled buffer without bovine serum albumin. 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 6000 × *g* for 12 min to pellet the thylakoids. For thorough Cl^- depletion, the thylakoids were resuspended and diluted to about 50 μg Chl per ml in a large volume of buffer containing 1.0 μM Gramicidin D (freshly added) and 20 mM bis-tris propane/morpholinepropanesulfonic acid (BTP/Mops) (pH 8.6) [13]. 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 per ml in 50 mM Hepes (pH 7.2). Thylakoid samples for ^{35}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. [14]. 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 100 mM sucrose in the grinding buffer. Thylakoids taken from the first high-speed centrifugation step were resuspended to 2.0 mg Chl per ml in buffer containing 5 mM $MgCl_2$, 2 mM $CaCl_2$, 2 mM sodium ascorbate (freshly added), and 20 mM morpholineethanesulfonic acid (pH 6.0). Triton X-100 (Calbiochem-Behring) was added to a final concentration of 25 mg per mg Chl. The suspension was stirred on ice in darkness for 25 min, transferred to centrifuge tubes, and centrifuged for 25 min at 40000 × *g*.

Chloride depletion of Photosystem II membranes. The green pellet (minus a very small grey impurity layer) 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^-. Treatment with sodium sulfate was found to be effective at removing residual Cl^- from the membranes (as determined by assaying activity in the absence of Cl^-), but also tended to lower slightly the maximum activity attainable in 50 mM Cl^-. For this reason, a brief pH-jump treatment was used instead [15]. 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) per ml 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, 400 mM sucrose at pH 5.5. The Cl^- depleted PS II membranes were centrifuged a final time and resuspended to 2.5 mg Chl per ml in the Cl^--free buffer (pH 6.0).

Finally, the sample was distributed into measured aliquots and frozen immediately in liquid N_2, where it was stored until use. Chl concentration was measured by a method discussed by Graan and Ort [16].

Hill-reaction measurements. The Hill activity of the thylakoids was measured by 2,6-dichlorophenolindophenol reduction. Thylakoids were suspended at 5.8 μg Chl per ml in buffer containing 1.0 μM gramicidin D, 50 μM DCIP, and 50 mM Hepes (pH 7.2), along with a measured amount of NaCl. We are aware that somewhat different results may have been obtained if we had chosen electron acceptors other than DCIP (see Sandusky and Yocum: Ref. 17). In order to minimize random errors in $[Cl^-]$, a set of assay buffers for the activation curves was prepared by proportional dilution of two assay buffer stocks, one without Cl^- and one with the maximal concentration of Cl^-. Thylakoids were allowed to incubate in the assay buffer in the cuvette for 3 min before illumination. The apparent level of Cl depletion (measured by comparing the Hill activity: ±50 mM Cl^-) was approx. 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 Ref. 18 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 carrousel 4200 slide projector, and was passed through a Corning C.S. 3-69 yellow filter (50% transmission at 525 nm) and 1 inch of aqueous 1% $CuSO_4$. The assay suspension contained 20 μg Chl per ml in buffer containing 400 mM sucrose, 3.0 mM potassium ferricyanide, 0.4 mM 2,6-dichlorobenzoquinone 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^- depletion for PS II membranes was approx. 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^- at the active site, the remaining pool of bound Cl^- eventually equilibrates with the Cl^- buffer. The time-scale of the NMR measurement is long enough for this phenomenon not to affect the binding curve.

The maximum activity for the PS II membranes in 50 mM Cl^- was found to vary over the course of a year (see Fig. 2, Ref. 19), with the lowest activity being observed during the summer (June to October). Such seasonal variations are well known for market spinach thylakoids and their expression in the PS II preparations is not surprising.

Sample preparation for NMR measurements. For ^{35}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 contents were loosened form 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 [15] indicated that Cl^- binds to groups on the membrane with a 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, higher than 99.999%) was purchased from Aldrich.

NMR measurements. ^{35}Cl-NMR measurements were carried out at 19°C on a home-built 250 MHz spectrometer using a specially designed sideways-spinning probe (see Ref. 20) capable of hold-

ing a 20 mm (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 24508 kHz using a 33 μs 90°-pulse and 360 ms recycle time. Signals were detected in quadrature with 32 K data points and a spectral width of $\pm$25000 Hz. They were then block averaged (500 scans per block) and transferred to a hard disk by a Nicolet 1180E computer. The data points were multiplied by an exponential line-broadening factor to reduce the noise. This added 15 Hz to the observed linewidth for samples in 0.1 mM to 0.5 mM Cl^- and 10 Hz to those in 0.6–20 mM Cl^-. The data were then Fourier-transformed into the frequency domain. The observed linewidth for each sample was measured at half-maximum intensity with a ruler and compared with a computer-fit of the lineshape. The linewidth for free Cl^- in solution was measured by taking the spectra of buffer solutions containing 10 mM Cl^-. There was no detectable concentration dependence of the linewidth for these solutions in the range of 0.1–10 mM Cl^-. Spectra of these standard solutions were recorded periodically in order to check the field homogeneity during the runs. The computer-calculated signal-to-noise ratio for a 10 mM Cl^- buffer standard after 1000 accumulations with no added line-broadening was 58 : 1. The net or excess linewidth ($\Delta\nu_t$) caused by thylakoids was calculated by subtracting the linewidth for the Cl^- buffer standard from the observed linewidth for the membrane suspension [12]. Values of $\Delta\nu_t$ obtained at each [Cl^-] for different sample preparations were averaged without normalization. Error bars represent the sample standard error of the mean.

Results

In order to determine the effect of 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 [Cl^-] at various light intensities (Fig. 1). At high light intensity, the activity increased, apparently, in a smooth hyperbola as the [Cl^-] is increased from 0 (no NaCl addition) to 50 mM. This is consistent

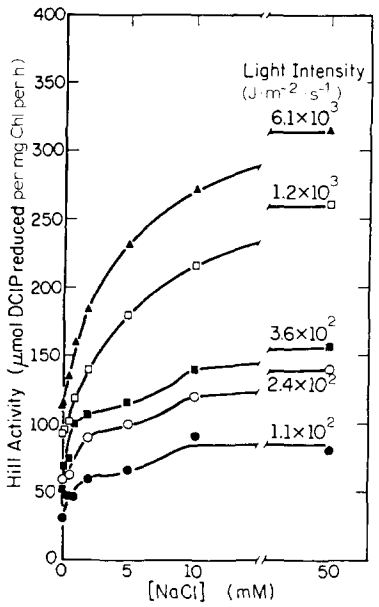

Fig. 1. Cl^- activation of the Hill reaction in thylakoids at various light intensities. The intensities listed with each curve represent (from top to bottom) 93%, 18%, 5%, 4% and 2% of saturating light intensity, respectively.

with earlier measurements of the effect of Cl^- on O_2 evolution [3]. However, at low light intensity (less than approx. 5.5% of saturation) the activation curves showed a non-hyperbolic dependence on [Cl^-]. (In contrast to the high-light, the low-light condition is closer to the 'dark' conditions of NMR measurements.) The deviations from hyperbolic behavior were most prominent at [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 mM Cl^-. In order to see the points more easily at low [Cl^-], these results are plotted as a function of [Cl^-] on a log scale (Fig. 2). This plot, which for a typical Michaelis–Menten system gives a smooth sigmoid [21], shows clear deviations from simple behavior. In this experiment we were able to observe at least three intermediary plateaus in the curve (centered at approx. 0.5 mM, 1.5 mM and 4.5 mM Cl^-) along with increases in slope (at approx. 0.75–0.9 mM, 2.5 mM and 5.0 mM Cl^-) and decreases in slope (at approx. 0.25–0.3 mM, 1.0 mM, 3.5 mM and 6.0–7.0 mM Cl^-).

It is therefore impossible to define a single-activator dissociation constant (K'_A) for Cl^- in

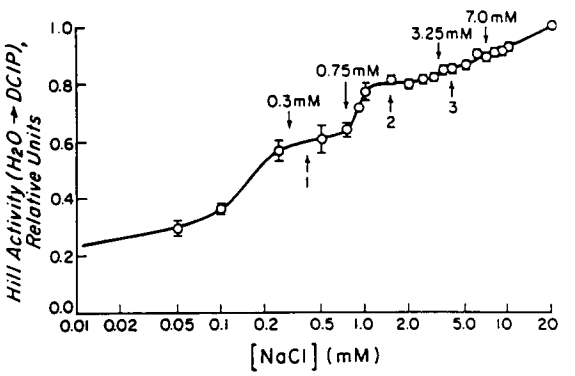

Fig. 2. Relative Hill activity vs. log [Cl^-] for spinach thylakoids at 5.5% of saturating light intensity (38 mW·cm^{-2}). Chloride concentrations marked with arrows (top) refer to corresponding points shown later in Fig. 5. The maximum activity (at 20 mM Cl^-) was 252 μmol DCIP reduced (per mg Chl per h). Error bars show the sample standard error for separate measurements on two different preparations.

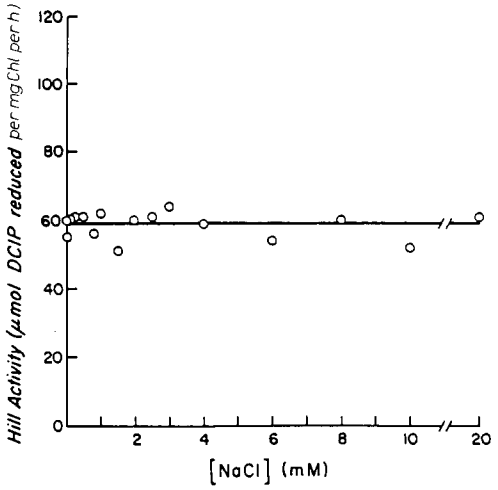

Fig. 3. Effect of Cl^- on the rate of electron flow from DPC to DCIP for thylakoids heated at 45°C for 5 min. The light intensity was 38 mW·cm^{-1}, the same as in Fig. 3. The DPC concentration was 1.0 mM. The assay pH was 7.2.

this system. The first three 'steps' in the curve contribute 47%, 27%, and 8%, respectively, to the amount of activity that is stimulated by Cl^- addition.

In thylakoids in which the OEC has been inactivated by heating, Cl^- has no effect on the diphenylcarbazide → DCIP reaction, indicating that at this light intensity, Cl^- does not effect the rate limitation on electron transport beyond the OEC (Fig. 3). DPC is known to donate electrons at Z, the electron donor to P-680$^+$, bypassing the OEC. This is consistent with the localization of the observed Cl^- effect on the donor side of PS II.

The appearance of intermediary plateaus (i.e., complex kinetic cooperativity) in the Cl^- activation curve for the Hill reaction indicates not only that multiple Cl^- binding sites might be involved, but also that Cl^- may bind cooperatively to these sites [22,23]. In order to investigate the Cl^- binding properties of the OEC by an independent and more direct method, we measured the Cl^- concentration dependence of the ^{35}Cl-NMR linewidth in both thylakoids and PS II membranes. For a system in which a Cl^- bound to a protein or other macromolecule exchanges rapidly with Cl^- in solution, the following equation can be written [12]:

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

where $\Delta\nu_{obs}$, the observed ^{35}Cl-NMR linewidth at half-maximum intensity, is the weighted average of the contributions from 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 Cl^- bound (f_b). The latter is not known, so we plot the net or excess linewidth ($\Delta\nu_t$), which depends on f_b. Moreover, $\Delta\nu_b$ (approx. 10 kHz) is very much larger than $\Delta\nu_f$ (approx. 12–30 Hz, depending on the viscosity), and $f_b \ll 1$ (for a dilute protein solution). Therefore, to a very good approximation:

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

Fig. 4 shows representative spectra used to determine $\Delta\nu_t$ for PS II membranes.

For a simple system, Eqn. 2 would be expected to produce a binding curve for $\Delta\nu_t$ vs. [Cl^-] in the form of a smoothly descending hyperbola, since f_b decreases with increasing [Cl^-]. This type of curve has been observed for many Cl^--binding enzymes, including those in which more than one Cl^- binding site is present (see Ref. 12 and NMR references in Ch. 1, sections F and H, in Ref. 19). We use the term 'binding curve' in an operational sense to distinguish the ^{35}Cl-NMR measurements from the kinetic (Hill activity) measurements of

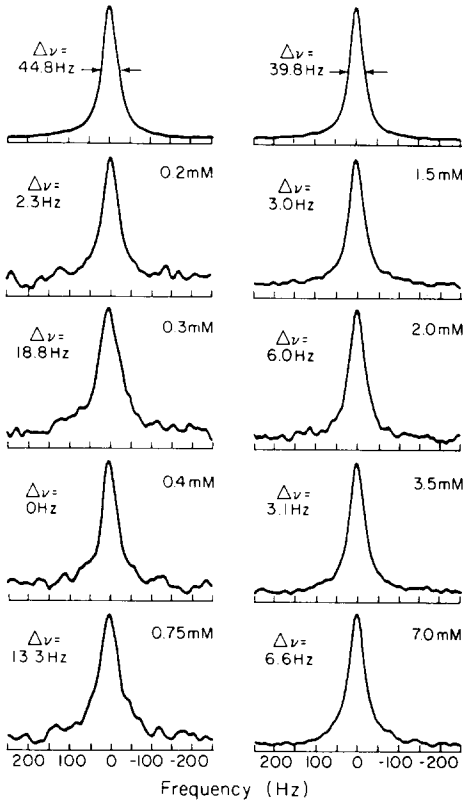

Fig. 4. Examples of ^{35}Cl-NMR spectra for PS II membranes at various Cl^- concentrations. The Chl concentration for these and other NMR measurements of PS II membranes was 0.5 mg·ml^{-1}. The top spectrum in each column represents a Cl^- buffer standard (10 mM NaCl in 400 mM sucrose, 20 mM Mes (pH 6.0), 1000 accumulations). The instrumental 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 24000 (0.2 mM Cl^-) to 2000 (7.0 mM Cl^-).

Figs. 1–3. The use of the term 'binding' is consistent with a similar usage in ligand binding to proteins (see Gill et al. [24]). The relative contributions to the NMR linewidth of quadrupolar relaxation and chemical exchange are not yet known in the spinach system. However, see Baianu et al. [12] for the halophyte system.

In Cl^- depleted thylakoids and PS II membranes, however, the ^{35}Cl-NMR binding curve is interrupted by sharp increases in linewidth in the concentration range between 0.1 mM and 10 mM Cl^-. As indicated by the measurements of the excess linewidth at each [Cl^-], the size of $\Delta\nu_t$ alternately decreases and increases several times as the [Cl^-] increases.

Fig. 5 shows the ^{35}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 details). It is clear from these measurements that the excess linewidth as a function of [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 Cl^-), the curve is interrupted by increases in linewidth at 0.3 mM, 0.75 mM, 3.25 mM, and 7.0 mM Cl^-. As may be seen in Fig. 2, these ^{35}Cl-NMR linewidth maxima occur at roughly the same [Cl^-] as the deviations in the Cl^- activation curve for the Hill activity. Differences in the location of the inflection points may reflect the difference in the temperature at which the two measurements were made (19°C for the NMR vs. 25°C for the Hill activity).

A similar phenomenon is observed in PS II membranes (Fig. 6). In this system, the overall linewidths are slightly narrower, probably as a

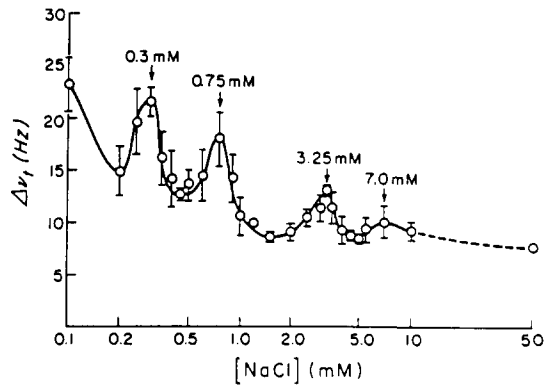

Fig. 5. ^{35}Cl-NMR binding curve for thylakoids. The mean Hill activity for all of the preparations (34 in all) was 343 μmol DCIP reduced (per mg Chl per h). The Chl concentration was 1.0 mg ml^{-1}. Each point with an error bar is the mean value for $\Delta\nu_t$ at a given [Cl^-] for 3–8 measurements 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) a slight variation in the concentration of endogenous Cl^-, which tends to shift the entire curve to the right or left; (2) inaccuracies in the measurement of $\Delta\nu_t$ (i.e., the Cl^- buffer standard), which shift the entire curve up or down; and (3) a variation in the activity of the preparations, which affects the magnitude of the linewidth maxima (see Fig. 7). Approx. 400 h of NMR instrument time (for both accumulation and processing) were required to obtain the complete curve.

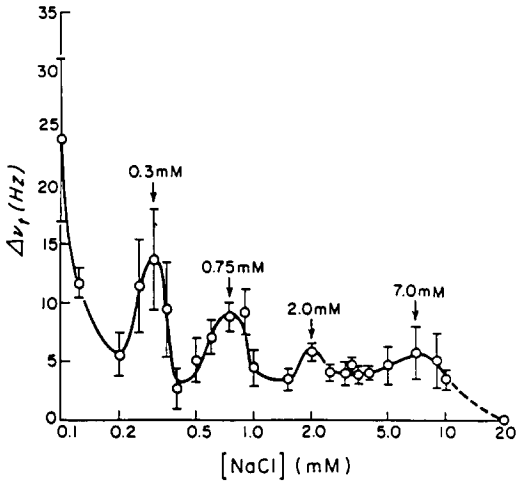

Fig. 6. ^{35}Cl-NMR binding curve for PS II membranes. The mean activity for all the preparations (10 in all) was 383 μmol O_2 per mg Chl per h. The Chl concentration was 0.5 mg·ml^{-1}.

result of the removal of the non-specific Cl^- binding sites present in thylakoids [12]. In these preparations, the linewidth maxima appear at 0.3 mM, 0.75 mM, 2.0 mM and 7.0 mM Cl^-. The reason for the apparent shift of the third maximum to lower [Cl^-] is not known. The ratio of the linewidths for the four linewidth maxima is approx. 5 : 3 : 2 : 1.5.

After measuring the ^{35}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. 7. Because of this trend, only the ^{35}Cl-NMR results from preparations with the highest activity have been used for Fig. 6.

As a check on whether the NMR results could be simply an instrumental artifact, we measured a ^{35}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. 8). For this protein, which contains a number of Cl^- binding sites, we found that $\Delta\nu_t$ shows little deviation from a linear dependence against log [Cl^-]. Previous ^{35}Cl-NMR studies of serum albumin at higher [Cl^-] have also failed to show any deviation from expected behavior [10].

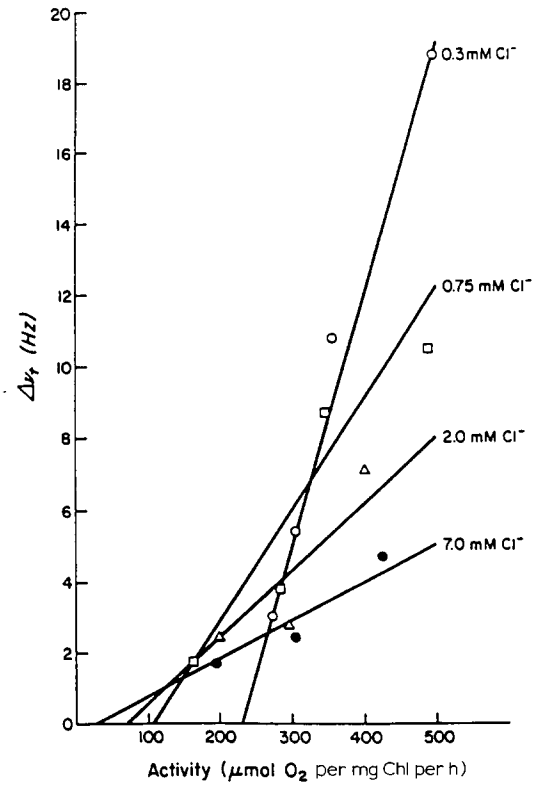

Fig. 7. Dependence of the four ^{35}Cl-NMR linewidth maxima (Fig. 6) on the OEC activity for PS II membranes. Different preparations over a period of 1 year, with different OEC activity, were used to construct this curve.

Br^- is known to activate O_2 evolution nearly as effectively as Cl^- [3,4], so we tested whether the excess ^{35}Cl line-broadening we have observed in PS II can be reduced by addition of a small, constant amount of Br^- (0.1 mM) to the PS II suspension. The resulting ^{35}Cl-NMR binding curve showed an overall decrease in excess linewidth

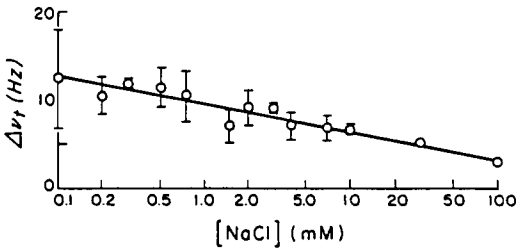

Fig. 8. ^{35}Cl-NMR binding curve for bovine serum albumin. The protein was dissolved at a concentration of 0.1 mg ml^{-1} in 50 mM Hepes at pH 7.2. Error bars show the sample standard error for 2–4 measurements of $\Delta\nu_t$.

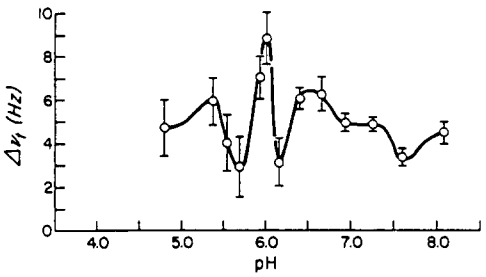

Fig. 9. pH dependence of $\Delta\nu_t$ at 0.75 mM Cl^-. The mean activity of the preparations (7 in all) was 338 μmol O_2 per mg Chl per h at pH 6.0. Error bars show the sample standard error for 3–7 measurements of $\Delta\nu_t$. The buffer concentration was 20 mM. In order to control the pH precisely, 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/phospahte 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.

and a substantial loss of line-broadening at the lowest [Cl^-] (data not shown; see Fig. 16, Ch. II in Ref. 19). This confirms that Br^- competes with Cl^- in this system.

Homann [15] has demonstrated that the binding of Cl^- to PS II membranes requires the protonation of a group having a pK_a of approx. 6.0. Therefore, we examined the pH dependence of $\Delta\nu_t$ at one of the linewidth maxima (0.75 mM Cl^-) to determine whether there is any correlation between the pH dependence for Cl^- binding and the pH dependence for Cl^- activation of O_2 evolution. Fig. 9 shows that the pH optimum for Cl^- binding at this [Cl^-] is also at 6.0. Interestingly, however, there are also two smaller peaks at approx. 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.

Discussion

The results presented here demonstrate that it is possible to measure Cl^- binding to the OEC in spinach thylakoids and PS II membranes by ^{35}Cl-NMR. However, the appearance of linewidth maxima in the ^{35}Cl-NMR binding curve (Figs. 5 and 6) cannot be explained by Eqns. 1 and 2, which assume that $\Delta\nu_b$ is a constant and that f_b decreases monotonically with increasing [Cl^-], and that fast exchange occurs between bound and free Cl^-. The only meaningful way to explain these sharp, Cl^--dependent increases in linewidth is to propose that the additional line-broadening arises from Cl^- binding to previously non-exchanging sites within the OEC, and that these sites are opened up to binding and/or exchange by the addition of Cl^-. This explanation implies that 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 Cl^- in the steady-state kinetics of activation (Figs. 1–2) lends support to this interpretation. Moreover, both measurements indicate that four distinct 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 Cl^- in thylakoids) contribute to these effects. The relative contribution of the maxima in the excess linewidth is approx. 5 : 3 : 2 : 1.5. Although mathematical analyses have been made of simple cooperativity in enzyme kinetics [22], they have yet to be extended to the more complex cooperativity exhibited by the Cl^- binding.

The ^{35}Cl-NMR linewidth maxima observed here are not simply the result of a non-specific ionic strength effect. Measurements reported in the accompanying paper [34] show that these same linewidth maxima appear in NaCl-washed PS II membranes in the presence of 2.0 mM $CaSO_4$.

In an earlier ^{35}Cl-NMR study of Cl^- binding to thylakoids from halophytes, Baianu et al. [12] did not observe any anomalies in a plot of $\Delta\nu_t$ vs. [Cl^-] at a Chl concentration of 2.9 mg · ml^{-1} for *Avicennia germinans*. However, since the Cl^- requirement of the halophyte thylakoids is much higher than in spinach [4], the details of the Cl^- binding must also differ. In particular, the NMR measurements of the halophytes were at Cl^- concentrations 10- to 1 000-fold higher than those employed here.

To our knowledge there are no other reports of sharp increases in ^{35}Cl-NMR line-broadening in a protein system, but an equivalent phenomenon has been reported for counter-ion binding to a synthetic polymer. By observing the binding of another quadrupolar nucleus, ^{23}Na, which binds to the polyanion polymethacrylic acid, Gustavsson et al. (Refs. 25 and 26; see also Lindman [27])

detected a large increase in the ^{23}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 Cl^- binding to protonatable groups within the OEC [28] may involve similar changes in conformation.

There is already substantial evidence to indicate that bound Cl^- in the OEC is tightly associated with a sequestered pool of protons [13,28,29]. Some of these protons are believed to be pumped into the sequestered domains by the light-driven oxidation of water [13]. Since the release of 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 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 Cl^-) by brief illumination of the thylakoids [29] then this would explain our unpublished data (Coleman [19]) showing that pre-illumination partially eliminates the inflections in the $H_2O \rightarrow$ DCIP Cl^- activation curve. The likely reason, as Homann [15] has suggested, is that H^+ must bind to sites on the OEC before Cl^- binding may proceed. The binding of Cl^- is thus limited by the concentration of H^+, its co-activator [23].

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 apparent cooperativity with respect to the [Cl^-] vanishes (Fig. 1. Ref. 3). A similar effect has been observed in CTP synthetase from *Escherichi coli*, which exhibits diminished cooperativity with respect to the concentration of substrate (glutamine) as the concentration of GTP (an allosteric activator) is raised [30].

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 (Refs. 7, 31 and 32; see also Chapter 1 in Ref. 19). 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^- [31]. An analogous situation may occur in the OEC. For example, the four linewidth maxima that we have observed in thylakoids and PS II membranes might reflect the existence of four sets of such salt-bridges. Cooperative differential binding of Cl^- in these four domains might explain both the different sizes of the linewidth maxima and the relative magnitude of each increase in O_2 evolution between plateaus in the Cl^- activation curve.

The pH dependence of Cl^- binding exhibits a maximum excess linewidth at pH 6.0, a value which is the same as Homann's determination of the pK_a from kinetic measurements [15]. However, unlike the pH-activity curve for O_2-evolution, which is a smooth bell-shape [5], the linewidth vs. pH curve has two minima and two smaller maxima flanking the mean peak at pH 6.0. Homann has suggested that the apparent pK_a of 6.0 for Cl^- activation of O_2 evolution is compatible with the possible involvement of histidyl residues in Cl^- binding. The data in Fig. 9 would also be consistent with the involvement of 1–3 such histidyl groups in controlling the movement of Cl^- and H^+ within the OEC. For a detailed discussion of a working hypothesis for the mechanism of the role of Cl^- in OEC, see Ref. 33.

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