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NMR study of chloride ion interactions with thylakoid membranes

(photosynthesis/oxygen evolution/chloride binding/chlorine-35/halophytes)

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ABSTRACT **The role of Cl^- in photosynthetic O_2 evolution has been investigated by observing the ^{35}Cl NMR linewidth under a variety of conditions in aqueous suspensions of chloroplasts, primarily for the halophytes *Avicennia germinans*, *Avicennia marina*, and *Aster tripolium* but also for spinach. The line broadening shows there is weak, ionic binding of Cl^- to thylakoids, the bound Cl^- exchanging rapidly ($>>10^4$ sec^{-1}) with free Cl^- in solution. The binding is necessary for O_2 evolution to occur. Michaelis–Menten constants obtained from the Cl^- dependence of the O_2 evolution rate are ≈15–70 mM for the halophytes compared with 0.6 mM for spinach (0.5 mM with Br^-). There appear to be two types of Cl^- binding sites in halophytes, of which the stronger is the activator, at lower [Cl^-], of O_2 evolution. The ^{35}Cl line broadening includes a nonspecific interaction, which becomes apparent at high Cl^- concentrations (≳0.5 M).**

The chloride ion is an essential cofactor for photosynthetic O_2 evolution (1–4), but the mechanism of its action has not been established. The addition of 10–20 mM Cl^- to Cl^--depleted thylakoids from glycophytes restores almost completely their O_2-evolving activity (1–3). For halophytes, the [Cl^-] needed is >10-fold higher (5, 6). Moreover, only anions with similar ionic radii (Br^-, NO_3^-) are effective in replacing Cl^-. Small anions such as F^- and OH^- are inhibitory, F^- probably by displacing Cl^-, while larger ions have no effect (6, 7).

The large Cl^- requirement (0.1–0.5 M) makes the halophytic thylakoids particularly suitable for ^{35}Cl NMR studies (6, 7). We have reported elsewhere (6, 7) that O_2 evolution in the presence of optimal Cl^- concentrations has a pH dependence very similar to that of the ^{35}Cl NMR line broadening, which is indicative of Cl^- binding. This observation and the specificity for Cl^- have led us to propose that Cl^- facilitates electron transport in photosystem II (PSII) by reversible ionic binding affecting the O_2-evolving complex or the thylakoid membrane nearby (7).

In the present paper we consider in more detail the dependence of the Hill reaction rate upon [Cl^-] and its implications with respect to the binding and role of the Cl^-. We describe the ^{35}Cl NMR results for thylakoids from two halophytic species, *Avicennia* (*Avi.*) *germinans* and *Aster* (*A.*) *tripolium*, including line shapes and their dependence upon Cl^- and thylakoid concentrations and upon temperature. The line broadening is used to determine the equilibrium constant K_b for the binding of Cl^- to the thylakoids. In turn, an estimate of the binding energy is obtained from the values of K_b at two different temperatures. The broadening mechanism is discussed as is its relationship to the dependence of the Hill activity upon [Cl^-]. In addition, we report the effect of F^- upon the Cl^- line broadening and our observation with a sideways spinning sample of the ^{35}Cl and ^{81}Br NMR line shapes at low [Cl^-] and [Br^-] in thylakoids of *Spinacia* (*S.*) *oleracea* (spinach).

MATERIALS AND METHODS

Thylakoids and Hill Reaction. Thylakoids were isolated from leaves of *Avi. germinans* and *A. tripolium* as reported (7). The *A. tripolium* thylakoids were isolated from plants that were grown in salt-sufficient medium. Chlorophyll concentration [Chl] was determined as described elsewhere (8). Electron transport activity was measured polarographically as O_2 evolution with ferricyanide, except for our spinach data in Table 1, which were determined spectrophotometrically with 2,6-dichlorophenolindophenol as electron acceptor at saturating light intensity (7). Thylakoid preparation and Cl^- depletion were carried out in spinach as described by Kelley and Izawa (3) and also by a method that uses an uncoupler of photophosphorylation at alkaline pH (9). All reaction rates were measured at 25°C.

NMR Spectra. The ^{35}Cl NMR spectra were recorded at 24.51 MHz on a laboratory-assembled, high-field multinuclear spectrometer (NSF 250) of the Oldfield and Meadows design (10). ^{81}Br NMR spectra were recorded on the same spectrometer at 67.57 MHz under conditions similar to those for ^{35}Cl. Data accumulation and processing were performed on an NIC 1080 computer (Nicolet Instruments, Madison, WI). Standard conditions included accumulation of free induction decays in quadrature with 30-μs (90°) pulses, 102-ms recycling time, ±20 kHz sweepwidth, and 16-k data points. For optimal resolution, standard 5-mm NMR tubes were used with 3-mm inserts and ≈40 Hz vertical sample spinning. An NMR probe with a large sideways spinning sample tube (10) was used for ^{35}Cl and ^{81}Br spectra of spinach thylakoids with low anion concentrations. Unless otherwise specified, spectra were recorded at 25°C.

The linewidths usually are the average of measurements made graphically on several different recorded spectra. In some instances they are a measurement from the best of several spectra.

EXPERIMENTAL RESULTS

Hill Reaction Rates. The dependence of the Hill reaction rate upon [Cl^-] has been reported for thylakoids from several plant species. Our results for the halophytes *Avi. germinans* (figures 1 and 2 in ref. 7) and *Avicennia* (*Avi.*) *marina* (figure 1 of ref. 5) are compared in Fig. 1 with recent data of Izawa *et al.* (figure 2 of ref. 2) for spinach. The Hill activities are given in terms of the maximal reaction rate V_{max} for each set of data. The [Cl^-] scale for spinach is 100 times that for the halophytes and the data points obtained beyond 1.7 and 170 mM Cl^-, respectively, are not shown. The concentrations given include any residual Cl^- in the thylakoids used (7).

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Abbreviations: Chl, chlorophyll; PSII, photosystem II.

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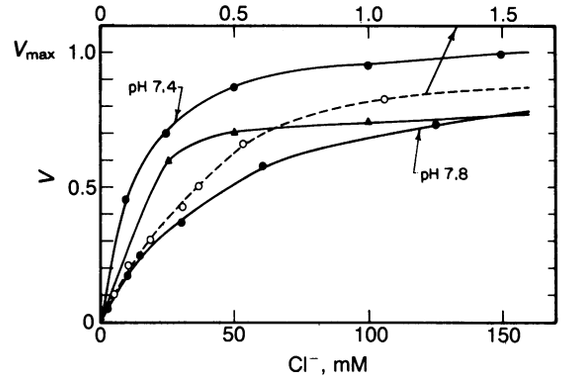

FIG. 1. The dependence upon total [Cl^-] of the Hill reaction rates, normalized to the maximal rate, for thylakoids from halophytic (bottom scale) and glycophytic (top scale) plants. ●, *Avi. germinans*; ▲, *Avi. marina*; ○, *S. oleracea*. Details are summarized in Table 1.

The Cl^- concentrations of 15–70 mM required by the halophytes for their $V_{max}/2$ rates are 20- to 100-fold larger than the ≈0.6 mM Cl^- (or Br^-) required by spinach. Also, at a pH of 7.8, *Avi. germinans* required nearly a 4-fold higher [Cl^-] for $V_{max}/2$ than at a pH of 7.4. Table 1 gives a summary of the Cl^--dependence data from Fig. 1 and from our current work on *A. tripolium* and spinach. The latter includes a set of measurements with added NaBr instead of NaCl. The Cl^- concentrations given for V_{max} are sometimes rather arbitrary; moreover, the Hill activity often decreases at still higher [Cl^-].

^{35}Cl NMR Line Shapes. Some representative ^{35}Cl line shapes are reproduced in Fig. 2; Fig. 2, curve E, is the spectrum of aqueous 0.5 M NaCl for comparison. It is a relatively narrow, Lorentzian line with a full width at half-maximal amplitude ($\Delta\nu$) of 17 Hz; this includes instrumental broadening of ≈3 Hz. The spectra of the thylakoid suspensions are severalfold broader. Such broadening is characteristic of the binding of Cl^- to proteins (11).

The spectrum of thylakoids from *Avi. germinans* with 250 mM NaCl (Fig. 2, curve A) is also a single Lorentzian line, with a $\Delta\nu$ of 86 Hz. However, those of *A. tripolium* also with 250 mM NaCl (Fig. 2, curve B) and of spinach with 10 mM NaCl (Fig. 2, curve C), besides having a central component similar to the spectrum of *Avi. germinans*, also have a symmetrically placed broader component visible in the shoulders of the main peak. For *A. tripolium* the widths of the two components are about 50 and 190 Hz, with integrated intensities of ≈2:3. For spinach at 10 mM NaCl the linewidths are 70 and 220 Hz, with intensities of 3:5.

By using rf probes having a sideways spinning sample (7 ml in vol) we are now able to record spectra of spinach thylakoids at 4 mg of Chl per ml with [Cl^-] as low as 0.2–0.5 mM

Table 1. Dependence of the Hill activity upon [Cl^-] for several different species of thylakoids and sets of conditions

Species	pH	Chl, μg/ml	$V_{max}/2$*	V_{max}*
Avi. germinans (7)	7.4†	30	14	150
Avi. germinans (7)	7.8	30	50	400
Avi. marina (5)	7.8	20–25	20	500
A. tripolium (7)	7.8	30	70	250
A. tripolium	7.2	30	55	200
S. oleracea	7.2	30	0.5‡	70‡
S. oleracea (2)	7.4	10	0.35 (0.6)§	25 (70)§

References are in parentheses.
*Cl^- concentrations are shown in mM.
†Prepared at pH 7.1 but measured at pH 7.4.
‡With Br^- instead of Cl^-.
§Obtained as described in *Analysis of Results*.

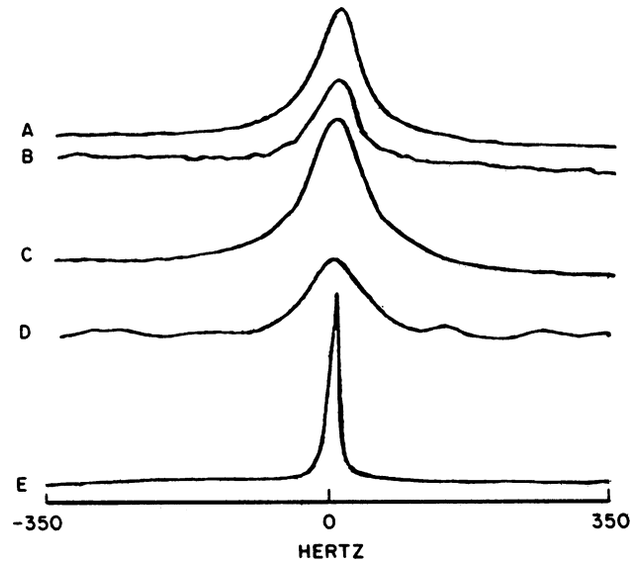

FIG. 2. ^{35}Cl NMR line shapes recorded at 24.51 MHz for 5°C with 4000 scans. Curve A, thylakoids of *Avi. germinans* with 250 mM NaCl, 50 mM Tricine, 1 mM $MgCl_2$, and 2.9 mg of Chl per ml at pH 7.4. Curve B, *A. tripolium* with 250 mM NaCl, 50 mM phosphate buffer, and 3.1 mg of Chl per ml at pH 7.2. Curve C, spinach with 10 mM NaCl, 50 mM phosphate buffer, and 3.1 mg of Chl per ml at pH 7.2. Curve D, spinach prepared with 0.2 mM NaCl (9), 4 mg of Chl per ml at pH 7.2, and an uncoupler of photophosphorylation. Curve E, 0.5 M NaCl in H_2O (200 scans).

(Fig. 2, curve D). At these low Cl^- concentrations only a single peak was seen; the $\Delta\nu$ of that in Fig. 2, curve D, is 170 Hz.

Changes in ^{35}Cl Linewidth. The width of the single, Lorentzian ^{35}Cl NMR line we observed in thylakoid suspensions under most conditions is affected by several parameters, including pH, [Cl^-], [Chl], and temperature. The pH dependence of $\Delta\nu$ for *Avi. germinans* and *A. tripolium* has been reported elsewhere (7). The dependence upon [Cl^-] of the additional broadening $\Delta\nu_t$ caused by thylakoids of *Avi. germinans* is given in Fig. 3 for 2.9 and 5.0 mg of Chl per ml, 25°C and a pH of 7.2. We define $\Delta\nu_t$ as

$$\Delta\nu_t = \Delta\nu_{obs} - \Delta\nu_{free}, \qquad [1]$$

in which $\Delta\nu_{obs}$ is the linewidth observed in the thylakoid suspensions and $\Delta\nu_{free}$ is that for "free" Cl^- in aqueous NaCl at the same total [Cl^-].

At both Chl concentrations in Fig. 3, $\Delta\nu_t$ is largest for low

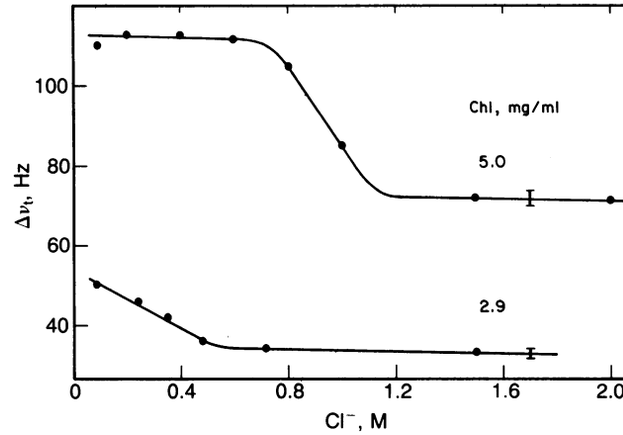

FIG. 3. Dependence upon [Cl^-] of ^{35}Cl line broadening $\Delta\nu_t$ by thylakoids of *Avi. germinans* at pH 7.2, 25°C, 2.9 and 5.0 mg of Chl per ml, and buffer as for Fig. 2, curve A.

[Cl^-], decreases with increasing [Cl^-], but "flattens out" to a virtually constant value at high [Cl^-]. The inflection point is 0.55 M Cl^- for 2.9 mg of Chl per ml and 0.95 M for 5 mg of Chl per ml, reflecting the dependence of $\Delta\nu_t$ upon [Chl]. The dependence of $\Delta\nu_t$ upon [Chl] at fixed Cl^- (0.6 M) is shown in Fig. 4 for 5°C; it is linear except possibly for concentrations above ≈6 mg/ml.

The effect of temperature (0–17°C) upon $\Delta\nu_t$ is shown in Fig. 5 for thylakoids of *Avi. germinans* at pH values of 7.2 and 5.8 with 2.9 mg of Chl per ml and 1 M NaCl. The broadening is less at the smaller pH and increases for both at lower temperatures. The increase is linear in $1/T$ between 7°C and 17°C but deviates to larger $\Delta\nu_t$ at lower temperatures. In these experiments the line shape was a single Lorentzian at pH 7.2 for all temperatures. However, at pH 5.8 an incompletely resolved and broader peak was observed below ≈7°C, when the [Chl] was >5 mg/ml. The center of the broader component appeared to have the same chemical shift as the narrow component and the NaCl solution.

Changes Induced by Fluoride. The central components observed in the ^{35}Cl resonance for *A. tripolium* and spinach (Fig. 2, curves B and C), with 250 and 10 mM NaCl, 3.1 mg of Chl per ml, and pH 7.2, were about 50 and 70 Hz, respectively. Inclusion of 250 mM NaF in these suspensions and incubation of them for 10 min in the dark prior to measurement narrowed the lines to about 35 Hz for both suspensions (by 30% and 50%). Under the same conditions, the NaF reduced the O_2 evolution by 50% in *A. tripolium* and 90% in spinach. Also, the broad ^{35}Cl component decreased in intensity in both cases.

ANALYSIS OF RESULTS

Hill Reaction Rates. Fig. 1 shows that the Hill activity of glycophytic and halophytic thylakoids increases with the [Cl^-]. As pointed out by Kelley and Izawa (3), Cl^- activates in some manner the O_2-evolving enzyme (E) of PSII. The simplest sort of schematic model for the activation is as follows

$$E + Cl^- \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ECl^- \xrightarrow[k_p]{h\nu,\ H_2O} E + Cl^- + O_2 + 4H^+ + 4e^-. \quad [2]$$

The kinetics of O_2 evolution by such a process are the same as the initial reaction rate of a unireactant enzyme for which Cl^- is the (hypothetical) substrate and O_2 the product (12). If the [Cl^-] >> [E], the dependence of the Hill activity upon [Cl^-] should be given by the Henri–Michaelis–Menten equation

$$v/V_{max} = [Cl^-]/(K_m + [Cl^-]). \quad [3]$$

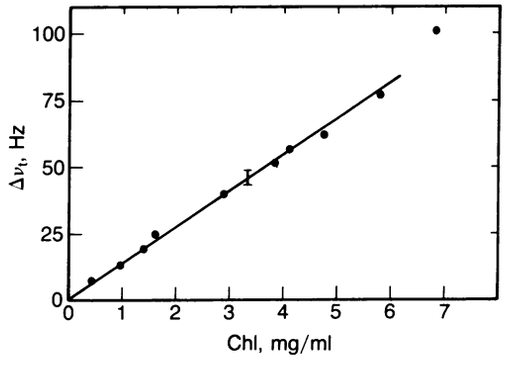

FIG. 4. Dependence upon [Chl] of ^{35}Cl line broadening $\Delta\nu_t$ by thylakoids of *Avi. germinans* at pH 7.4, 5°C, 0.6 M NaCl, and buffer as for Fig. 2, curve A.

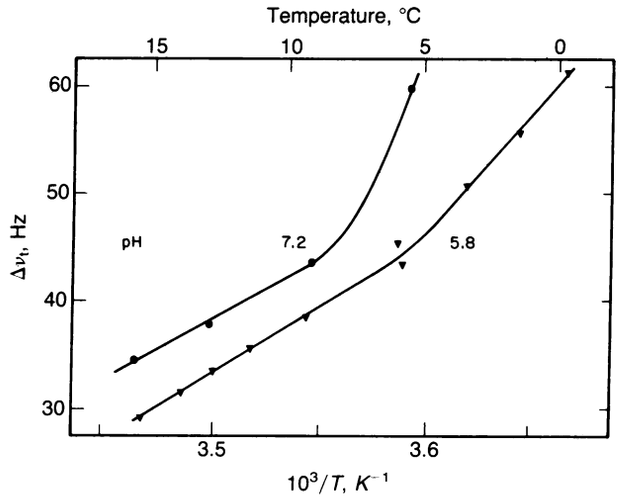

FIG. 5. Temperature dependence of ^{35}Cl line broadening $\Delta\nu_t$ by thylakoids of *Avi. germinans* at pH values of 7.2 and 5.8, with 1 M NaCl, 2.9 mg of Chl per ml, and a buffer of 50 mM Hepes/1 mM $MgCl_2$.

For reaction **2** the Michaelis–Menten constant is

$$K_m = (k_{-1} + k_p)/k_1. \quad [4]$$

In the above model, if the ECl^- complex does not dissociate upon evolving oxygen, k_p drops out and K_m becomes the dissociation constant for the complex.

The curves in Fig. 1 are of the general form predicted by the Henri–Michaelis–Menten equation. However, the data were not obtained to establish the enzyme kinetics and are not optimum for the purpose. The best fit to the Henri–Michaelis–Menten equation is given by recent results for spinach (2). They are presented in Fig. 6 as a double-reciprocal plot of 1/Hill activity versus 1/[Cl^-], the latter including endogenous Cl^-. The $1/v$ data have been renormalized to a V_{max} that is 35% larger than the rate reported for 25 mM Cl^- (2). This gives a self-consistent linear plot in which the same value of 0.6 mM is obtained for K_m from the $1/v = 0$ intercept and from the [Cl^-] at $V_{max}/2$, as predicted by Eq. **3**. The K_m from the [Cl^-] for Izawa's $V_{max}/2$ is only 0.35 mM (Table 1). An earlier analysis by Kelley and Izawa (3) of spinach data gave a larger K_m, 0.9 mM at pH 7.2. However, the medium contained 2 mg of bovine serum albumin per ml, which might bind Cl^-.

A linear double-reciprocal plot (not shown), with a K_m of 50 mM, was obtained from the data in Fig. 1 for *Avi. germinans* at pH 7.8. However, the double-reciprocal plots of the data for the other entries in Table 1 exhibit curvature. For

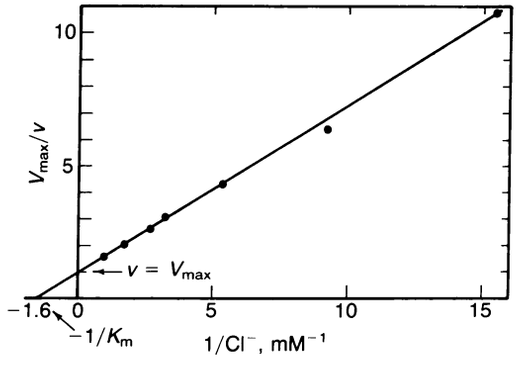

FIG. 6. Double-reciprocal plot of $1/O_2$-evolution rate (v), normalized to V_{max} (see text), versus 1/[Cl^-], using data of Izawa *et al.* (2) for *S. oleracea* at 21°C with 10 μg of Chl per ml.

them, we list the Cl^- concentrations at $V_{max}/2$ obtained from the curves in Fig. 1. They should be approximate values of K_m for the systems studied.

^{35}Cl NMR Line Shapes. In the thylakoid suspensions the ^{35}Cl and ^{81}Br NMR line shapes are broadened by quadrupolar relaxation associated with binding of the anions to proteins in the membranes (11, 13–15). By adding EDTA to the suspensions and by decoupling the protons and using ^2H_2O instead of H_2O as the medium, we have shown that relaxation by paramagnetic species (7) and by proton dipolar interactions contributes <1–2 Hz to the ^{35}Cl linewidth. Therefore, a ^{35}Cl nucleus bound to a thylakoid is predicted to have a Lorentzian line shape with a width determined by the quadrupolar interactions (11)

$$\Delta\nu_b = (2/5)\pi(e^2qQ/\hbar)^2\tau_c, \quad [5]$$

in which e is the electronic charge, eq is the electric field gradient at the nucleus, which has an electric quadrupole moment eQ, and τ_c is the correlation time characterizing fluctuations of the electric field gradient.

Fast chemical exchange of Cl^- between bound and free (but hydrated) states averages the different relaxation rates and linewidths and gives in the extreme narrowing limit (11, 15) a single Lorentzian line of width

$$\Delta\nu_{obs} = f_b\,\Delta\nu_b + (1 - f_b)\Delta\nu_{free}, \quad [6]$$

in which f_b is the fraction of Cl^- that is bound. $\Delta\nu_b$ can be quite large so that the binding of even a small fraction of the Cl^- can affect the averaged linewidth by substantial amounts. Such averaging accounts for the single ^{35}Cl NMR lines found in most of our observations (Figs. 2–5).

However, composite line shapes similar to those in Fig. 2, curves B and C, were found in several instances. They tend to occur at higher Chl concentrations and acidic pHs—for example, in *Avi. germinans* at [Chl] ≳6 mg/ml and pH 5.8. A study was made of the temperature dependence of the composite line shape for the latter. Above 7°C the line shape became single and the temperature dependence of its width paralleled that for the single line for *Avi. germinans* in the less concentrated suspension (2.9 mg of Chl per ml, Fig. 5), also at pH 5.8. This suggests that the broad component arises from the separation under some conditions of the suspension into two "phases" between which the Cl^- exchange is slow.

At the high Chl concentrations employed, the suspensions are semisolid, so such behavior is not unreasonable. A likely explanation is that the broad component arises from Cl^- "trapped" in and more tightly bound to the grana stacks of appressed membranes. Electron microscopy has shown that under the conditions in which the broader ^{35}Cl component is observed, there is also a larger proportion of appressed membranes (refs. 16–18, glycophytes; unpublished data, halophytes). One would expect Cl^- diffusion through the grana stacks to be slower than through unappressed thylakoids.

Temperature Dependence of ^{35}Cl Linewidth. Our analysis of the *concentration* dependence of the ^{35}Cl linewidth is based on Eq. **6**, which assumes that chemical exchange is fast enough for the extreme narrowing limit to apply. Usually $\Delta\nu_b >> \Delta\nu_{free}$ (11), also $f_b << 1$, so to a good approximation, Eqs. **6** and **1** can be combined as

$$\Delta\nu_t = \Delta\nu_{obs} - \Delta\nu_{free} \cong f_b\Delta\nu_b. \quad [7]$$

When chemical exchange is slow, the value for $\Delta\nu_b$ in Eq. **7** is replaced by the more general term (14)

$$1/(T_{2b} + \tau_{ex}) = 1/[(1/\pi\Delta\nu_b) + \tau_{ex}], \quad [8]$$

in which T_{2b} is the transverse relaxation time of chlorine nuclei in the bound state, τ_{ex} is their mean lifetime there, and $\Delta\nu_b$ is still that given by Eq. **5**.

The lifetime τ_{ex} decreases at higher temperature, so if it were significant the broadening $\Delta\nu_t$ produced by the thylakoids would increase at higher temperatures. Alternatively, if τ_{ex} is negligible, the temperature dependence of $\Delta\nu_t$ is determined by that of τ_c in Eq. **5**, which decreases at higher temperatures. In the actual case, $\Delta\nu_t$ decreases at higher temperatures (Fig. 5), confirming that the ^{35}Cl linewidth is dominated by the quadrupole relaxation. This conclusion is consistent with the detailed studies of Cl^- binding to hemoglobin (13).

Concentration Dependence of ^{35}Cl Linewidth. If the fraction f_b of the Cl^- bound to thylakoids is small, it may be expressed in terms of the binding constant K_b, the total concentration of binding sites $[E]_t$, and the total Cl^- concentration $[Cl^-]$. Thereby, Eq. **7** is converted to the following expression (13) for the concentration dependence of the resultant broadening

$$\Delta\nu_t = \Delta\nu_b K_b[E]_t/(1 + K_b[Cl^-]). \quad [9]$$

For a fixed $[Cl^-]$ it predicts that $\Delta\nu_t$ will be a linear function of $[E]_t$ and thereby of [Chl]. This agrees with the data in Fig. 4, where the straight line extrapolates to the origin.

For fixed [Chl], Eq. **9** predicts that $\Delta\nu_t$ is largest at low $[Cl^-]$ and drops to zero hyperbolically as the $[Cl^-]$ becomes large and the fraction bound becomes small. The data for *Avi. germinans* in Fig. 3 at Chl concentrations of 2.9 and 5.0 mg/ml are in only partial agreement with this prediction. At high $[Cl^-]$ both curves approach relatively large $\Delta\nu_t$s, ≈30 and ≈70 Hz, respectively, rather than zero. These results are similar to those reported for chloride binding to hemoglobin, which were interpreted in terms of two binding sites, one characterized by a high and the other by a low binding constant (13).

In our case, the near constancy of $\Delta\nu_t$ at high $[Cl^-]$ leads us to suggest that it is a limiting value caused by a nonspecific interaction of the Cl^- with the membranes. The relaxation mechanism could be the diffusional encounter by Cl^- ions of the hydrophobic hydration spheres of nonpolar groups in the membranes, such as that proposed to account for the strong relaxation of ^{81}Br in aqueous solution with organic solutes (19). Support for this interpretation is provided by the effects of heating the thylakoids for 3.5 min at 45°C (7). The treatment eliminates oxygen evolution and reduces the ^{35}Cl line broadening to the ≈30 Hz found at higher $[Cl^-]$ with unheated thylakoids, both attributable to deactivation of the specific binding sites by heating.

Another feature of Fig. 3 at variance with Eq. **9** is the "plateau" region of large $\Delta\nu_t$ at the lower $[Cl^-]$ in the top curve for 5 mg of Chl per ml. Moreover, the top curve is displaced upward from the bottom curve by more than the predicted ratio of Chl concentrations (5:2.9). The discrepancies most likely are caused in some manner by the large [Chl], as in Fig. 4. The curves analyzed to obtain values for the binding constant were for lower [Chl], which did not exhibit this behavior.

K_b may be estimated graphically from the dependence of $\Delta\nu_t$ upon $[Cl^-]$. We subtract the limiting value, $\Delta\nu_{lim}$, of $\Delta\nu_t$ found at high Cl^- from the $\Delta\nu_t$ observed at lower Cl^-, defining

$$\Delta\nu_t' = \Delta\nu_t - \Delta\nu_{lim}. \quad [10]$$

The $\Delta\nu_t'$ should be the net line broadening caused by the binding of the Cl^- to specific sites of the thylakoids. Introducing it into Eq. **9** and rearranging it gives

$$\Delta\nu_b[E]_t(1/\Delta\nu_t') = [Cl^-] + (1/K_b). \quad [11]$$

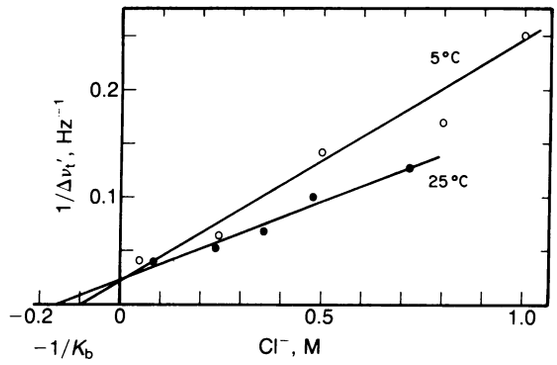

FIG. 7. The inverse ($1/\Delta\nu_t'$) of the broadening of the ^{35}Cl NMR linewidth, excluding that from the nonspecific interactions (see text) plotted versus [Cl^-] for suspensions of thylakoids from *Avi. germinans*, 2.9 mg of Chl per ml, buffer as in Fig. 2, curve A.

Accordingly, the reciprocal of the net line broadening should be a linear function of [Cl^-], for which the [Cl^-] intercept is $-(1/K_b)$ and the slope is $1/(\Delta\nu_b[E]_t)$.

Plots of this type are given in Fig. 7 for suspensions of *Avi. germinans* at 5°C and 25°C. They were obtained by adjusting $\Delta\nu_{lim}$ to give straight lines, the values used being 54 and 27 Hz, respectively. The intercepts extrapolated for $1/\Delta\nu_t' = 0$ give approximate binding constants for the Cl^- of 10 and 7 M^{-1}, also for 5°C and 25°C, respectively. The difference in K_b at these two temperatures corresponds to a binding energy ΔE_b of about 3 kcal/mol (1 cal = 4.184 J).

At this point we consider the concentration of binding sites $[E]_t$. The data at hand are insufficient in themselves to determine $[E]_t$ but we can make a rough estimate. $[E]_t$ appears as a product with $\Delta\nu_b$, the linewidth for bound Cl^-, in the expression $1/\Delta\nu_b[E]_t$ for the slopes of the lines in Fig. 7. Our results for K_b are comparable with those (10 M^{-1} at 22–24°C) reported for the binding of Cl^- to hemoglobin (13). In the latter, $[E]_t$ was known and thereby the concentration-dependence studies gave a value for the $\Delta\nu_b$ of hemoglobin of $\approx 3 \times 10^4$ Hz, a result that should be approximately applicable to the thylakoids. The slope of the line in Fig. 7 for 25°C is $\approx 0.15\ Hz^{-1}\cdot M^{-1}$. Combining these two values leads to an $[E]_t$ of ≈0.2 mM. The Chl concentration of 3 mg/ml is 3.3 mM, which gives one binding site per 16 Chl (25 Cl^- per 400 Chl). This binding ratio is about four times that found necessary for activating the O_2-evolving system in spinach thylakoids, using the radioactive isotope ^{36}Cl (9).

The estimates of 0.2 mM for $[E]_t$ and 3×10^4 Hz for $\Delta\nu_b$ confirm the assumptions made earlier in the analysis that [Cl^-] >> $[E]_t$, that $f_b << 1$, and that $\Delta\nu_b >> \Delta\nu_{free}$. Also, the value for $\Delta\nu_b$ places a lower bound of $3 \times 10^4\ s^{-1}$ upon the exchange rate of the bound Cl^-.

DISCUSSION

A consistent, though not yet complete, picture emerges from these results. The ^{35}Cl NMR data show that in aqueous suspensions of glycophytic as well as halophytic thylakoids, there is weak ionic binding of Cl^- to the membranes, the bound Cl^- exchanging rapidly with the free Cl^- in solution. The binding of Cl^- or Br^- is necessary to activate the O_2-evolving enzyme or to otherwise enable O_2 evolution to occur. Displacement of the Cl^- by F^- prevents the O_2 evolution. With spinach, the K_m for Br^- is the same as that for Cl^-.

The ^{35}Cl NMR line broadening for halophytic thylakoids includes a nonspecific interaction, or very weak binding, which becomes apparent at high [Cl^-] (≳0.5 M). The specific binding found at lower [Cl^-] occurs in two different concentration regions. With *Avi. germinans*, the [Cl^-] range from ≈100 mM to 2 M was observed by the line-broadening experiments and that from 0 to ≈100 mM was observed by Hill activity measurements. The Cl^- binding constant K_b from the former [Figs. 3 (curve at 2.9 mg of Chl per ml) and 7] is $\approx 7\ M^{-1}$. In the case of the Hill activity, the reciprocal of K_m should be a lower bound to K_b, giving a value of $\gtrsim 70\ M^{-1}$.

Thus, it appears that in halophytes there are two types of Cl^- binding sites, of which the stronger is the main or only activator of O_2 evolution, while the weaker causes the line broadening observed at higher [Cl^-]. However, as shown by the $\Delta\nu_t$ of 153 Hz for 0.2 mM Cl^- in spinach (Fig. 2, curve D), the ^{35}Cl line broadening accompanying the activation of O_2 evolution can be observed at low [Cl^-].

These results shed little light upon the detailed mechanism by which binding of Cl^- activates the O_2 evolution. Earlier work located the site of Cl^- action on the electron donor side of PSII and led to speculations that the fast reversible binding of Cl^- might be necessary for the advancement of the S states (2–4, 7). Izawa *et al.* (2), in reporting flash experiments on chemically treated thylakoids, have pointed to Cl^- dependence of the $S_1 \rightarrow S_2$ step. Thus far, sensitivity limitations have restricted the ^{35}Cl line-broadening measurements to Cl^- concentrations well above those at which O_2 evolution is activated. Recent improvements in NMR instruments should enable observation of the linewidths at lower Cl^- and Chl concentrations for better comparison with the Cl^- dependence of the O_2 evolution rates.

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