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^{35}Cl-NMR linewidth measurements of aqueous suspensions of Photosystem II membrane fragments reveal only a simple hyperbolic dependence with chloride concentration

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It has been reported that measurements of the excess ^{35}Cl-NMR linewidth for dark-adapted aqueous suspensions of Photosystem II membrane fragments show as many as four sharp, peak-like increases in the linewidth values superimposed upon a hyperbolically declining background over the 0.1–10 mM chloride concentration range (Coleman, W.J., Govindjee and Gutowsky, H.S. (1987) Biochim. Biophys. Acta 894, 443–452). Similar peak-like increases in the ^{35}Cl linewidth values can also be observed when a Varian XL-400 NMR instrument is employed. However, although the peak-like increases are sensitive to the physiological state of the Photosystem II samples, the phenomena that are observed using the Varian instrument are actually a consequence of a systematic instrumental irregularity. Upon employing a Bruker WB-360 NMR instrument, in which the systematic irregularity is less or non-existent, the ^{35}Cl linewidth values for aqueous suspensions of Photosystem II membrane fragments show a simple hyperbolic decline with increasing chloride concentrations. The data indicate that only a single class of exchangeable chloride interaction sites having a relatively high binding affinity is detectable in Photosystem II by NMR.

Introduction

The mechanism by which water is photooxidized to O_2 in Photosystem II (PS II) of chlorophyll *a*-containing plants is largely unknown. However, manganese ions are an absolute requirement, while a large body of literature suggests that the presence of chloride (or other substituting anions) is essential as well (for a recent review, see Ref. 1). Although chloride ions have been proposed to act as a direct cofactor in the water-oxidizing reactions [2–7], it may be that the effects of chloride on the O_2 evolution activity are only indirectly mediated through the long-range conformational status of the PS II complex [8]. Nevertheless, in order to understand the role of chloride in O_2 evolution, it is necessary to have an experimental method which can directly monitor chloride interactions with PS II.

The use of ^{35}Cl-NMR linewidth measurements to determine exchangeable chloride binding to macromolecular complexes in aqueous media is well documented (see Ref. 9). However, in comparison with other forms of spectroscopy, NMR has a relatively low sensitivity. The established NMR measurements of exchangeable chloride binding to macromolecular complexes have been made with samples containing millimolar-range concentrations of binding sites at chloride solution concentrations in excess of 10 mM. In these cases, the linewidth enhancements are measured in the order of tens to hundreds of hertz. For PS II samples, which even in the most concentrated form probably contain less than 100 μM binding sites, the contributions of any exchangeable chloride binding to the linewidth will be considerably less. But with improvements in instrument sensitivity and techniques to increase S/N, it should be possible to monitor exchangeable chloride binding to PS II by NMR at sufficiently low chloride concentrations.

Abbreviations: Chl, chlorophyll; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PS II, Photosystem II; $\Delta\nu_{1/2}$, linewidth value at half-height.

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In the limit of fast exchange, the behavior of the excess ^{35}Cl-NMR linewidth in an aqueous medium containing a macromolecular complex which binds chloride is expected to decline in a hyperbolic fashion with increasing chloride concentrations. This has been shown, for example, in the case of chloride binding to concentrated cytochrome *c* samples at relatively high chloride concentrations (i.e., 20–1000 mM) [10]. In contrast, linewidth measurements of PS II samples at chloride concentrations less than 10 mM have been reported to show rather sharp, peak-like increases in the linewidth values superimposed upon a hyperbolically declining background [11,12]. The sharp, peak-like increases were found to be specific (as many as four in the 0.1–10 mM concentration range) and were interpreted by the authors to indicate different (co-operative) chloride binding domains within the PS II complex.

In our first measurements of the chloride concentration dependence of the ^{35}Cl-NMR linewidth of PS II membrane fragments using a Varian XL-400 instrument, we also observed the sharp, peak-like increases. But it became apparent later in our work that these linewidth phenomena actually arose from a systematic instrumental irregularity. Upon employing a Bruker WB-360 instrument, in which the instrumental irregularity was less or non-existent, the ^{35}Cl-NMR linewidth of the PS II membrane fragments showed only a simple hyperbolic decline with increasing chloride concentrations. In this communication we report our findings.

Experimental

Samples of Triton X-100 prepared PS II membrane fragments were made from either market or hydroponically grown spinach as described earlier [14]. After the detergent solubilization, the membrane fragments were washed three times at a sample concentration of roughly 0.5 mg Chl/ml in a buffer medium consisting of 20 mM Mes/NaOH (pH 6.3), 400 mM sucrose, 15 mM NaCl and 5 mM $MgCl_2$ in order to remove the excess detergent. After this treatment, the membrane fragments were pelleted by centrifugation at $12000 \times g$ for 5–10 min, leaving a colorless supernatant. The membrane fragments were then stored in the same buffer medium in concentrated form (5–7 mg Chl/ml) as small beads frozen in liquid nitrogen. The initial steady-state rates of O_2 evolution were between 400 and 600 μmol O_2/mg Chl per h when assayed in the same buffer medium containing 2 mM $K_3Fe(CN)_6$ and 0.2 mM phenyl-*p*-benzoquinone as the electron acceptors.

Immediately prior to the NMR measurements, the frozen PS II membrane fragments were quickly thawed and washed twice in a medium consisting of only 20 mM Mes/NaOH (pH 6.3) at a 30–40-fold dilution (i.e., a sample concentration of 50–100 μg Chl/ml) in order to remove the excess chloride. After the second wash, the samples were resuspended to 5 mg Chl/ml in the same buffer medium and kept on ice until diluted into the final suspension medium used during the measurements. All sample manipulations were carried out in the dark or with a weak green safe light at 4° C or on ice.

For Tris-inhibited samples, the PS II membrane fragments were first incubated in 0.8 M Tris-HCl (pH 8.2) at 100 μg Chl/ml for 30 min on ice with constant stirring in dim room light. The samples were then centrifuged and washed twice to remove the excess chloride as described above. For thermally inactivated samples, the PS II membrane fragments, after being washed to remove the excess chloride, were allowed to incubate at room temperature in the dark at 500 μg Chl/ml for 80 h. Neither the Tris-inhibited nor the thermally inactivated samples showed any steady-state O_2 evolution activity.

The ^{35}Cl-NMR linewidth measurements (except those shown in Fig. 1a) were made using either a Varian XL-400 instrument (39.1 MHz resonance frequency) located in the Department of Chemistry, Chalmers University of Technology, or a Bruker WB-360 instrument (35.2 MHz resonance frequency) located in the Iwan-Stranski Institute, Technical University Berlin. The operating conditions for the Varian instrument were: 90° pulse, 80 μs; acquisition time, 0.096 s; recycle time, 0.256 s; sweep width, 2000 Hz; filter, 2.2 kHz; digital resolution, 5.2 Hz/point; receiver gain, 13; magnetic field, unlocked (non-spinning sample); NMR tube size, 1 cm diameter (3 ml sample volume); temperature, 20° C. The operating conditions for the Bruker instrument were: 90° pulse, 85 μs; acquisition time, 0.260 s; recycle time, 1.0 s; sweep width, 2000 Hz; filter, 2.5 kHz; digital resolution, 3.9 Hz/point; receiver gain, 1600; magnetic field, locked on a deuterium signal (10% D_2O was added to each sample) (non-spinning sample); NMR tube size, 2 cm diameter (20 ml sample volume); temperature, 22–24° C. The ^{35}Cl nuclear longitudinal relaxation time (T_1) is about 30 ms.

In both instruments, the signal free induction decays, detected in quadrature, were block averaged over 2000 scans (at 10 mM chloride) to 60000 scans (at 0.25 mM chloride using the Varian instrument and at 0.50 mM chloride using the Bruker instrument) and zero-filled to 16 K. After Fourier transformation, in each instrument, a single chloride line was obtained and fitted to a Lorentzian-shaped curve from which the linewidth at half-height was calculated by the computer using the standard fitting routines provided with each instrument. A 5 Hz line broadening function was used in the analysis of all measurements, except as noted in the text. For a 10 mM NaCl solution after 2000 scans, the linewidth reproducibility was $\pm$ 0.2 Hz and the S/N about 70 in the Varian instrument, while the linewidth reproducibility was $\pm$ 0.4 Hz and the S/N about 70 in the Bruker instrument.

For all measurements of the PS II samples, the washed PS II membrane fragments were diluted 10-fold to the final sample concentration of 500 μg Chl/ml. However, for the measurements shown in Fig. 1b and c, the samples were diluted directly into the buffer medium containing the appropriate chloride concentrations, while for the measurements shown in Fig. 4, the samples were first diluted into the chloride-free buffer medium to which aliquots from either a 100 mM or 1 M NaCl stock solution were added to give the appropriate final chloride concentration (maximum 1% volume change). Although the O_2 evolution activity of the PS II membrane fragments in the presence of chloride is relatively stable up to 20 h at room temperature in the dark (see Ref. 8), at the low chloride concentrations where long NMR measurement times were needed, freshly prepared sample aliquots were replaced at approx. every 2 h.

Results

Fig. 1a shows the ^{35}Cl-NMR linewidth data for aqueous suspensions of PS II membrane fragments reported in Fig. 6 from Ref. 11, replotted on a linear concentration scale. With increasing chloride concentrations, these data show not only a general decrease in the linewidth values, but also quite dramatic, peak-like increases at specific chloride concentrations. In our first measurements of aqueous suspensions of PS II membrane fragments using a Varian XL-400 instrument under comparable conditions, we also observed the peak-like increases in the linewidth values, although not exactly at the same chloride concentrations as reported in the earlier study. These data are shown in Fig. 1b. Similar to the earlier study, we also observed that the pattern of the peak-like, linewidth increases could be altered upon various perturbations to the sample. An example of Tris-inhibited PS II membrane fragments is shown in Fig. 1c, but the patterns could also be variously altered depending upon the temperature, pH or addition of divalent salts (e.g., $MgSO_4$).

The excess linewidth data presented in Fig. 1 are calculated by subtracting the linewidth value for the background solution form the measured linewidth in the presence of the sample. Theoretically, the linewidth for the background solution should be independent of the chloride concentration. Indeed, when we initially measured the background solution at a few chloride concentrations, the linewidth appeared to be constant. However, upon measuring the linewidth values of the background solution over many chloride concentrations using the Varian instrument, we were surprised to find that systematic linewidth fluctuations were present. The results from the linewidth measurements of a simple NaCl solution as a function of concentration are shown in Fig. 2a. Comparison between Fig. 2a and Fig. 1b

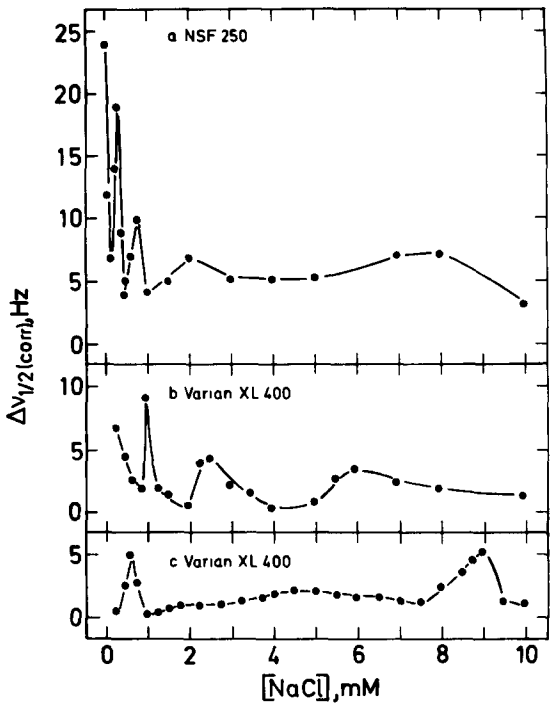

Fig. 1. Plots of the excess ^{35}Cl-NMR linewidth ($\Delta\nu_{1/2(corr)}$) for dark-adapted aqueous suspensions of Photosystem II membrane fragments as a function of NaCl concentration. The excess linewidth values were calculated by subtracting the value for a 10 mM NaCl background solution from the measured linewidth at each point. All measurements were made at a sample concentration of 500 μg Chl/ml. (a) Data for an O_2-evolving PS II preparation measured on a NSF 250 instrument, replotted from Fig. 6 in Ref. 11. The suspension medium consisted of 20 mM Mes/NaOH (pH 6.0), 0.4 M sucrose plus the added NaCl. The samples were pretreated to remove excess chloride using a brief pH jump at pH 8.2 (see Ref. 11). (b) Data for an O_2-evolving PS II preparation measured on a Varian XL-400 instrument. The suspension medium consisted of 20 mM Mes/NaOH (pH 6.3) plus the added NaCl. The samples were pretreated to remove the excess chloride as described in Experimental. (c) Data for a Tris-inhibited PS II preparation measured on a Varian XL-400 instrument. The suspension medium and sample pretreatment to remove the excess chloride were the same as used in (b). The operating conditions for the measurements shown in (a) are given in Ref. 11, while the operating conditions for the measurements shown in (b) and (c) are given in Experimental.

reveals that the linewidth fluctuations in the NaCl solution are about the same order of magnitude as the linewidth increases observed in the samples of the PS II membrane fragments, although the relative position of the peaks in the linewidth values with respect to the chloride concentration are shifted (all NMR instrumental parameters were kept identical for these measurements).

The instrumental parameters for the Varian XL 400 measurements were initially optimized for a 10 mM NaCl solution and only the number of scans was increased in order to obtain the data at the lower chloride concentrations with a comparable *S/N*. However, for concentrations higher than 10 mM, where even the

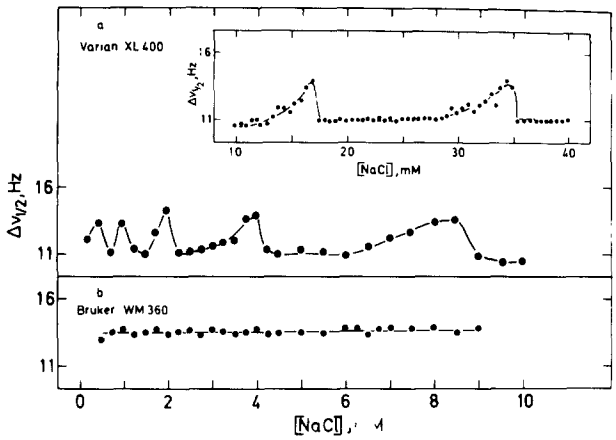

Fig. 2. Plots of the measured ^{35}Cl-NMR linewidth ($\Delta\nu_{1/2}$) for an aqueous background solution as a function of NaCl concentration. (a) Data obtained with a Varian XL-400 instrument. (b) Data obtained with a Bruker WB-360 instrument. Operating conditions are given in Experimental.

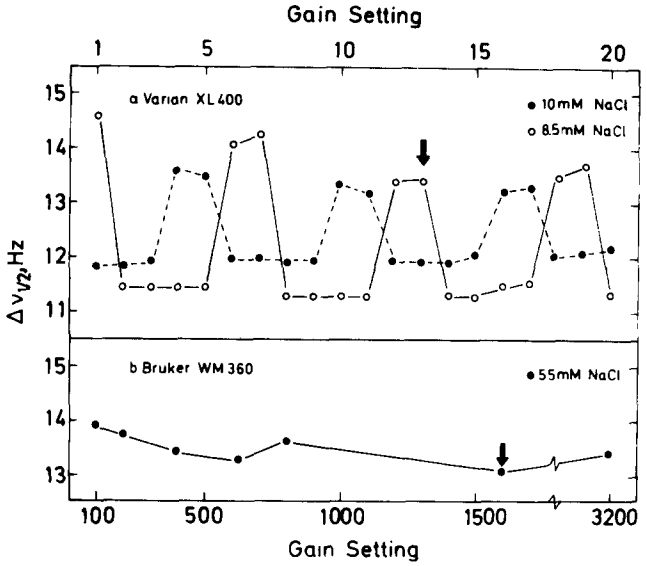

Fig. 3. Plots of the measured ^{35}Cl-NMR linewidth ($\Delta\nu_{1/2}$) for aqueous NaCl background solutions as a function of gain setting. (a) Data obtained with a Varian XL-400 instrument. (b) Data obtained with a Bruker WB-360 instrument. Other operating conditions are given in Experimental. The arrow indicate the gain settings used in the corresponding measurements shown in Fig. 1b and c, Fig. 2 and Fig. 4.

number of scans was held constant, the linewidth fluctuations were still present in the measurements of the NaCl solution. This is shown by the data in the inset of Fig. 2a, over the 10–40 mM concentration range. The data in Fig. 2a indicate that the peaks in the linewidth fluctuations occur at approximately every doubling of the chloride concentration (i.e., at about 0.5, 1, 2, 4, 8, 16 and 32 mM NaCl). Nevertheless, as shown in the inset of Fig. 2a, the linewidth values are virtually constant over the 18–28 mM range. This indicates that the Varian instrument is stable and that the linewidth fluctuations are not a random phenomenon. Rather, there appears to be a systematic irregularity, in at least the particular instrument which we used.

The linewidth fluctuations in the Varian instrument could not be overcome by adjusting: (1) the number of scans; (2) the digital resolution (by changing either the memory block size or the sweep width); (3) the receiver gating; (4) the acquisition time; (5) the transmitter offset; (6) the probe tuning; (7) the field homogeneity (with or without spinning); (8) the reference carrier phase used in quadrature detection; (9) the resonance frequency (e.g., the linewidth fluctuations were present whether natural abundance ^{35}Cl at 39.1 MHz or natural abundance ^{37}Cl at 35 MHz was measured); or (10) the size or sample volume of the NMR tubes. The linewidth fluctuations remained whether the linewidths were calculated manually from the chloride lines or through the standard fitting routines by the computer. However, when the r.f. pulse width was decreased or when a different preexponential function (i.e., a line-broadening term which is typically used to smooth the data points prior to Fourier transformation) was added during the data analysis, the observed peaks in the linewidth fluctuations would shift relative to the chloride concentration. This behavior would indicate that some form of lineshape distortion in relation to the absolute signal amplitude is somehow involved in the phenomena, although the NMR spectra gave no obvious indications of unusual distortions.

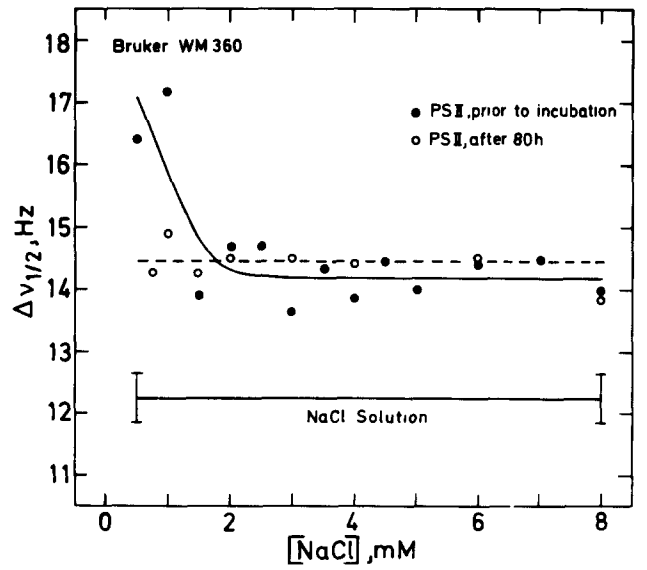

Fig. 4. Plots of the measured ^{35}Cl-NMR linewidth ($\Delta\nu_{1/2}$) for dark-adapted aqueous suspensions of Photosystem II membrane fragments, either active in O_2 evolution (●) or thermally inactivated (○), as a function of NaCl concentration. The suspension medium consisted of 20 mM Mes/NaOH (pH 6.3) and 20 μM EDTA plus the added NaCl at a sample concentration of 500 μg Chl/ml. The samples were pretreated to remove the excess chloride as described in Experimental. The data were obtained from a Bruker WB-360 instrument. Operating conditions are given in Experimental.

Fig. 3a shows the linewidth values measured by the Varian instrument for two NaCl solutions at different concentrations as a function of gain setting. In the Varian instrument the gain can be manually adjusted in steps from 1 to 59. The arrow shown in the figure indicates the gain setting chosen by the auto-gain option, which was used in all of the previous measurements. It is apparent from the data in Fig. 3a that the linewidth value varies with a distinct periodicity as a function of gain, with the phase of the periodicity depending upon the chloride concentration (or absolute signal amplitude). This behavior would account for the linewidth fluctuations as a function of chloride concentration when all instrumental parameters are held constant.

Upon using a Bruker WB-360 instrument, we could not detect any instrumental irregularity which interfered with the linewidth measurements. This is demonstrated in Fig. 2b, where the linewidths of a NaCl solution measured over the 0.5–9.0 mm concentration range show no obvious, systematic fluctuations. Likewise, as shown in Fig. 3b, for the permitted gain settings in the Bruker instrument, there is no obvious periodic variation in the linewidth values as a function of gain.

When the linewidth measurements of aqueous suspensions of PS II membrane fragments were made using the Bruker instrument, the data shown in Fig. 4 were obtained. In this case, there are no unusual, peak-like increases in the linewidth values outside the data scatter. Instead, there is only a weak decline in the linewidth values at chloride concentrations below 1.5 mM. After inactivation of the O_2 evolution activity by incubating the PS II sample in chloride-free medium at room temperature in the dark for 80 h, the weak decline in the linewidth values is lost over the concentration range measured. These results are also shown in Fig. 4. For these measurements, 20 μM EDTA was included in the sample suspension medium in order to eliminate any paramagnetic contributions to the ^{35}Cl linewidth from Mn(II) ions in the sample, as has been previously observed [8].

Discussion

In this communication, we have shown that the sharp, peak-like increases in the ^{35}Cl-NMR linewidths measured as a function of chloride concentration for aqueous suspensions of PS II membrane fragments using a Varian XL-400 instrument (Fig. 1b) are a consequence of a systematic instrumental irregularity (Fig. 2a and Fig. 3a). Comparable measurements using a Bruker WB-360 instrument do not reveal such unusual linewidth phenomena outside of the normal data scatter (Fig. 2b, Fig. 3b and Fig. 4). Although earlier reports have described similar linewidth phenomena using a different NMR instrument [11,12], it is possible that an instrumental irregularity similar to that which we have found in our Varian instrument may also be involved (Fig. 1a). Linewidth fluctuations due to an instrumental irregularity could easily be overlooked if the concentration range over which the linewidths are measured in a background solution is not thoroughly controlled. Additionally, since the maximum linewidth fluctuations occur only over a few hertz, which is sufficient to distort the data from PS II samples, they could nevertheless be considered part of the random error, particularly in those studies where the excess linewidths are measured in the order of tens or hundreds of hertz.

Even though the linewidth fluctuations in our measurements using the Varian instrument are sensitive to the physiological state of the sample (Fig. 1c), it is unlikely that they are related to different co-operative binding domains in PS II, as has been suggested [11,12]. Rather, it may be that weak, and probably non-specific, interactions between the PS II membrane fragments and chloride in this concentration range do affect the linewidth, and hence the signal amplitude, but in such a way that only the linewidth fluctuations show apparent shifts or disappear altogether. In the latter case, the signal amplitude would never reach the crucial point where the doubling triggers the instrumental irregularity.

The linewidth measurements of the PS II membrane fragments using the Bruker instrument indicate that only a simple hyperbolic decline occurs with increasing chloride concentrations (Fig. 4), as would be expected if there are contributions due to exchangeable chloride binding to the sample. However, the hyperbolic decline is weak and becomes observable only at chloride concentrations less than 1.5–2 mM. Such behavior would be consistent with a small fraction of exchangeable chloride bound to PS II at a site (or sites) having a relatively high binding affinity. For PS II samples prepared and treated in the same way as in this study, the steady-state O_2 evolution activity reveals a chloride dependence with an apparent $K_b < 25$ μM [15]. Upon inactivation of O_2 evolution after incubation of the sample in a chloride-free medium at room temperature for 80 h, the weak decline in the linewidth values at the low chloride concentrations is no longer observable over the concentration range measured (Fig. 4). This could indicate that either the exchangeable chloride binding sites are lost or that the binding affinity has increased. However, the level of the linewidth values for the inactivated PS II samples is about the same as that for the O_2-evolving PS II samples above 2 mM chloride, which is about 2 Hz greater than the linewidth for the NaCl background solution. This excess linewidth induced by the PS II membrane fragments may only be dominated by viscosity effects, rather than by contributions from exchangeable chloride binding, which could account for no effects of NH_3 on the excess linewidth being ob-

served at relatively high chloride concentrations (i.e., 10 mM) [16] (if the assumption is made that NH_3 competitively displaces bound chloride from PS II). Nevertheless, there can also be large excess linewidths by PS II samples at all chloride concentrations caused by paramagnetic interactions with Mn(II) ions [8], unless these effects are compensated in some way, either by using very fresh samples or by the addition of small amounts of EDTA.

In conclusion, ^{35}Cl-NMR linewidth measurements of PS II membrane fragments can exhibit the normal behavior expected from exchangeable chloride binding provided that any systematic instrumental irregularities and paramagnetic effects are taken into consideration. Although the contributions from exchangeable chloride binding to PS II in the linewidth measurements are weak, it should be possible in the future to analyze the data in terms of the binding affinities, number of sites, degree of cooperativity and the influence of the physiological state.

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