Volume 254, number 1,2, 184–188 FEB 07542 August 1989

Chloride binding to photosystem II in the dark is in slow exchange

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Received 14 June 1989

We have studied the $^{35}Cl^-$ NMR line broadening in the presence of photosystem II (PS II) membranes from spinach in the dark. In the presence of NH_3 (which other work has shown to competitively inhibit chloride binding to PS II) we observed no decrease in $^{35}Cl^-$ linewidths. We conclude that binding of Cl^- to the O_2 evolving center in PS II in the dark (previously demonstrated by EPR) is in slow exchange on the NMR timescale. We assign the observed line broadening to interaction with non-specific binding sites and with free paramagnetics.

Photosystem II; Chloride; NMR, $^{35}Cl^-$

1. INTRODUCTION

It has long been known that chloride is required for photosynthetic oxygen evolution [1]. The site of action has been localized to photosystem II (PS II) at the water-oxidizing side [2,3]. The chloride interaction is competitively and reversibly inhibited by ammonia, by certain primary amines and also by fluoride [4–6]. Binding of Cl^- and inhibitors to the O_2 evolving center (OEC) has been studied by a variety of techniques including EPR [4,7,8], EXAFS [9,10], luminescence [11] and NMR [12–16] and has been extensively reviewed [17–19]. There is evidence that Cl^- depletion prevents photo-oxidation of the OEC from S_2 to S_3 [20,21] (where S_0, S_1, S_2, S_3, and S_4 are the oxidation states of the catalytic photocycle of the OEC). It is possible to probe the binding of Cl^- and other ligands to the OEC in both the S_1 (which is the state of the OEC in the dark) and S_2 states by EPR methods [7,8,22]. Such studies have shown that NH_3 binds to PS II in the S_1 state and that this binding is prevented by Cl^-, thus showing that Cl^- binding to the OEC takes place in the dark. Also, several ^{35}Cl NMR studies with thylakoids and PS II membranes have shown high-affinity chloride binding and apparent correlations between the induced line broadening of the $^{35}Cl^-$ signal in the dark and O_2 evolving activity in light [12–15]. However, another NMR study with mangrove PS II indicates that broadening by high-affinity sites only occurs after illumination [16], implying that Cl^- binding only contributes to broadening of the NMR signal when the OEC is in higher S states, contrary to the other NMR work. The purpose of this study is to determine whether the binding of Cl^- to the chloride-binding site in the OEC in the dark contributes substantially to observed ^{35}Cl NMR line broadening.

Proteins increase the linewidth of the $^{35}Cl^-$ NMR resonance through the rapid relaxation of $^{35}Cl^-$ nuclei when Cl^- is bound to the protein [23]. Rapid exchange between protein-bound and free-solution environments induced broadening in the whole signal (whereas slowly exchanging or non-labile Cl^- does not contribute to observed line broadening). In order for line broadening to be observed, the lifetime of residence for Cl^- in free solution must be less than the time for spin-spin relaxation of the ions in free solution (in our case about 50 ms). Since any site to which Cl^- binds with rapid exchange kinetics will contribute line broadening, it is important to distinguish specific line broadening (due to interaction at the Cl^--binding site) from non-specific broadening due

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Published by Elsevier Science Publishers B.V. (Biomedical Division)

to interaction with other sites. The optimum way to do this is to use a specific competitor of Cl^- binding. This separation of specific from non-specific Cl^--binding sites has been achieved in NMR studies of another membrane protein system, the anion transporter in red blood cell membranes [24–26]. For PS II, competitive inhibitors of the Cl^- binding required for O_2 evolution include NH_3, primary amines and F^- [4–6]. Cl^- can also be replaced to varying extents by Br^-, NO_3^-, I^- and some other anions [12]. NH_3 is the best candidate for our purposes for several reasons: (i) it has been shown to compete effectively ($K_I = 0.39$ mM, [6]) with Cl^- for binding to PS II; (ii) EPR evidence discussed above [7,8] shows that binding of NH_3 takes place in the dark and that this is inhibited by Cl^-; (iii) NH_3 and $NH_4^+$are much less likely to compete with Cl^- for binding to non-specific sites than are anions.

2. MATERIALS AND METHODS

O_2 evolving preparations of PS II-enriched membranes were isolated from leaves of market spinach (*Spinacea oleracea*) by using a modified version of the Berthold et al. procedure [27], as described previously [22]. PS II membranes were stored at 77 K suspended at 5–10 mg chlorophyll/ml in a buffer solution containing 20 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes)/NaOH, pH 6.0, 15 mM NaCl, and 30% (v/v) ethylene glycol. All steps in the isolation procedure and subsequent handling of PS II membranes were performed under a green safe light. This procedure yielded dark-adapted PS II membrane preparations [22], ensuring that the O_2 evolving centers were in the S_1 state prior to treatment with reagents [28,32]. Chlorophyll concentrations were determined spectrophotometrically by the method of Arnon [29].

For NMR experiments, frozen suspensions of PS II membranes were thawed in the dark and then resuspended in a buffer solution containing 100 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes) (titrated to pH 7.5 with NaOH) and $CaCl_2$ to establish the indicated Cl^- concentration. No cryoprotectant was present. The suspension of PS II membranes was equilibrated in this solution by 3 centrifugation and resuspension steps. Then, the suspension was divided into 2 identical portions and centrifuged again. One pellet was resuspended in the same buffer (control), while the other was resuspended in an NH_3-containing buffer consisting of 100 mM Hepes (titrated to pH 7.5 with NH_4OH) and $CaCl_2$ to establish the same Cl^- concentration present in the control suspension. Thus, the 2 buffers had the same ionic strength, pH and ion concentrations except that one contained 50 mM Na^+ and the other 50 mM NH_4^+ ($[NH_3] = 0.9$ mM). The final chlorophyll concentration of both suspensions, which were directly used in NMR experiments, was 1 mg/ml. It has been shown [7,8] that under these conditions NH_3 (and other primary amines) bind to the OEC in the dark at the Cl^--binding site. The control and NH_3-treated PS II membrane suspensions retained full O_2 evolution activity following handling and use in NMR measurements, as checked by comparison to aliquots that were stored on ice in the dark for the duration of the NMR experiment.

^{35}Cl NMR measurements were performed on a Bruker AM-360 wide bore spectrometer operating at 35.3 MHz using a broad band probe at 27–30°C. This elevated temperature was chosen to increase exchange rates so as to maximize the likelihood of observing contributions to line broadening from all binding sites while still maintaining O_2 evolving activity. Spectra were obtained from 9 to 10 ml samples of PS II membranes at 1 mg Chl/ml held in 20 mm NMR tubes without spinning. Each spectrum was an accumulation of 4000 scans and took 5.3 min to acquire. Samples remained well suspended during NMR measurements. After exponential multiplication (5 Hz) and Fourier transformation of the data, linewidth measurements were made from the spectra either by fitting peaks using spectrometer software or by measuring the width at half height directly. Lorentzian lineshapes were observed throughout.

3. RESULTS AND DISCUSSION

Since Cl^- binds to PS II in competition with NH_3 in the dark as well as in the light, we wished to determine whether this binding contributes to the observed linewidth of $^{35}Cl^-$ in the presence of PS II. To do this we washed and resuspended portions of the same preparation of PS II membranes either in NH_3-containing or NH_3-free buffer. ^{35}Cl NMR spectra were then acquired and the linewidths compared to the linewidths from the buffer alone, thus yielding the line broadening due to Cl^- binding in the dark. Fig.1 shows examples of such spectra and it can be seen that good signal-to-noise ratios (and hence accurate linewidths) can be obtained under these conditions in a short period. This is because of the rapid spin-lattice relaxation ($T_1 < 40$ ms) which allowed 750 scans/min to be acquired. Since NH_3 competitively displaces Cl^- from the OEC (the $[NH_3]$ we used is sufficient to displace >75% of the Cl^- as assessed by O_2 evolution activity [5] and EPR data also indicates extensive displacement of Cl^- from S_1 under such conditions [8]) we would expect the observed $^{35}Cl^-$ linewidth to be less in the NH_3 buffer if binding of Cl^- to the Cl^--binding site in the OEC makes a contribution to the linewidth in the dark. As can be seen in fig.1, the line broadening in the NH_3-containing buffer (linewidth D minus linewidth C is 16 Hz) is more, not less than the line broadening in the NH_3-free buffer (linewidth B –

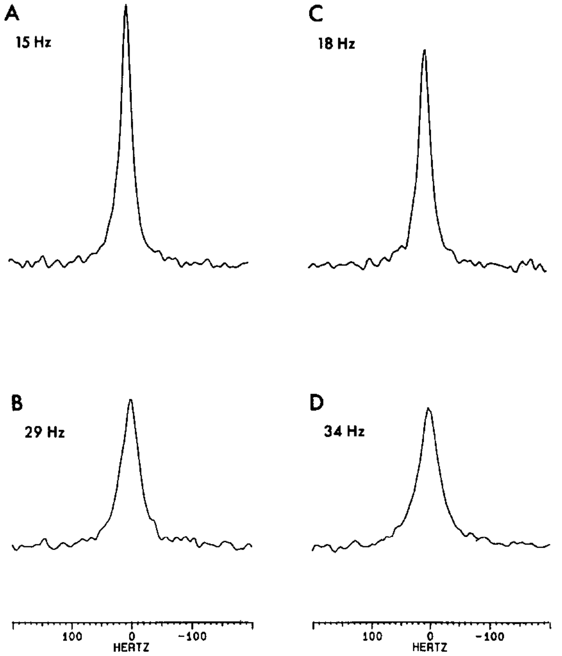

Fig.1. ^{35}Cl NMR spectra of PS II membrane samples and buffer solutions at 1 mM Cl$^-$. Linewidths are given for each spectrum as full width at half-height of the peak minus the 5 Hz line broadening added in processing. (A) NH_3-free buffer. (B) PS II membranes in NH_3-free buffer. (C) NH_3 buffer. (D) PS II membranes in NH_3 buffer.

linewidth A is 14 Hz). The results of this and other measurements are shown in table 1. Overall the line broadening by PS II was found to be 1.4 ± 1.9 Hz greater in the NH_3-containing than in the NH_3-free buffer (mean of 5 experiments ± SD). Thus, there is no significant difference between line broadening in the presence or absence of NH_3. There was a larger variation in the linewidths observed in different experiments with different PS II samples of the same chlorophyll concentration (1 mg · ml^{-1}) than one might perhaps expect, as was seen in other NMR studies [12–15]. However, the linewidths seen in the NH_3-containing and NH_3-free portions of the same PS II sample were always similar. Linewidths were observed with 0.5 and 1.0 mM Cl$^-$ and in neither case were significant differences seen between control and NH_4^+-containing samples. Since specific binding of Cl$^-$ to the OEC does not seem to contribute to the observed linewidth we next sought to determine what processes do contribute to line broadening in the dark in our spectra and in other studies [12–16].

3.1. *Broadening by non-specific anion binding sites*

Other competitors of Cl$^-$ binding to the OEC (Br$^-$ which can substitute for Cl$^-$ and F$^-$ which in-

Table 1

Summary of the results of ^{35}Cl NMR line broadening experiments

Line broadening results for the PS II experiments are quoted as the ^{35}Cl linewidth in the PS II sample minus the linewidth in the relevant buffer without membranes. The difference ± NH_3 in each measurement is the difference between the line broadening in the 2 matched PS II samples with and without NH_3

Preparation	[Cl]	[EDTA]	Na buffer	NH_3 buffer	Difference
Line broadening (Hz)					
PS II-1	1.0 mM	0	14	16	+ 2
PS II-2	0.5 mM	0	9	8	− 1
PS II-2[a]	1.0 mM	0	16	20	+ 4
PS II-3	0.5 mM	2.0 mM	3	5	+ 2
PS II-3	1.0 mM	2.0 mM	4	4	+ 0
					1.4 ± 1.9
Linewidths (Hz)					
BSA (− F$^-$)	10 mM	0		70	20
BSA (+ 250 mM F$^-$)	10 mM	0		50	
NaCl (1 μM Mn^{2+})	1.0 mM	0		22	10
NaCl (1 μM Mn^{2+})	1.0 mM	1.0 mM		12	
NaCl (1 μM Mn^{2+})	1 M	0		13	1
NaCl (1 μM Mn^{2+})	1 M	1.0 mM		12	

[a] In this case the [Cl$^-$] was raised from 0.5 to 1.0 mM by the addition of $CaCl_2$ (see text)

hibits) were observed by Coleman et al. [13] to reduce ^{35}Cl line broadening in PS II samples with Br^- giving a larger effect. These results were taken to indicate that the ^{35}Cl NMR line broadening by PS II is due to binding of Cl^- to the Cl^--binding site of the OEC. However, anions would be expected to compete with Cl^- for non-specific binding sites also. Norne et al. [30] observed reductions in the ^{35}Cl linewidth when Br^-, F^- or other anions were added to samples of human serum albumin [30]. Since serum albumins have no specific Cl^--binding sites, competition with Cl^- for non-specific binding sites was responsible. In that study high anion concentrations were used and rather small linewidth reductions were seen for F^-. To assess whether competition by anions for non-specific chloride sites can cause significant linewidth reductions under the conditions of PS II studies, we used bovine serum albumin with the same halide concentrations as those used in [13] with spinach PS II samples. When 250 mM F^- was added to a solution of BSA (in 25 mM Hepes at pH 7.4) containing 10 mM Cl^- line broadening by BSA was reduced from 70 to 50 Hz (table 1). This reduction in linewidth was smaller than observed with PS II [13] but of the same order of magnitude (being much larger than seen at high ionic concentrations [30]). This result shows that Cl^- binding to non-specific anion binding sites may be contributing significantly to ^{35}Cl line broadening by PS II.

3.2. *Broadening by paramagnetics*

In one of the PS II measurements we increased the [Cl^-] from 0.5 to 1.0 mM by the addition of $CaCl_2$ and observed a dramatic increase in linewidth (this measurement is indicated by an asterisk in table 1). Since Ca^{2+} is known to displace non-functional Mn^{2+} from loosely bound sites on the PS II membranes, we sought to address the possibility that such displacement of bound Mn^{2+} may be contributing to Cl^- linewidths. We used EDTA to chelate Mn^{2+} (and any other paramagnetic metal cations). In the presence of EDTA, line broadening decreased markedly (table 1) indicating that a significant fraction of the line broadening was due to free paramagnetic cations (this was supported by the observation of EDTA-sensitive line broadening in the supernatant from PS II samples that were incubated at 38°C for 10 min and then pelleted by centrifugation). In the presence of EDTA, the line broadening while less, was still very similar in the NH_3-containing and NH_3-free buffers (see table 1). This contribution from free paramagnetic ions (presumably Mn^{2+}) is in apparent disagreement with the observation by Coleman et al. [13] that in PS II samples from mangrove at 1 M Cl^- the linewidths were not significantly reduced (at most 1-2 Hz) by EDTA. (In that and other studies [12-15], no EDTA-containing control is quoted for samples at low Cl^- concentrations though Preston and Pace [16] performed all their experiments with EDTA present.) However, one would expect line broadening by Mn^{2+} to be more effective at low Cl^- concentrations; we, therefore, compared line broadening by Mn^{2+} in low and high [Cl^-] samples. We found that 1 μM Mn^{2+} caused ~10 Hz of EDTA-sensitive line broadening when [Cl^-] was 1 mM, but only 1 Hz of broadening when [Cl^-] was 1 M (see table 1).

4. CONCLUSIONS

Our observation of Lorentzian lineshapes throughout is consistent with one pool of Cl^- in the fast motional narrowing regime [23] in exchange with one or more class of labile sites. The observation of two co-resonant lines of different linewidths by Coleman et al. [13] at higher [Cl^-] may be due in some cases to the bi-exponential relaxation which spin 3/2 nuclei can undergo [23] rather than to 2 pools of Cl^-. A Lorentzian lineshape may also be observed in the presence of two groups of binding sites, the exchange with one being rapid and with the other slow enough that it contributes a negligible amount to the observed linewidth.

The finding that NH_3 binding to the OEC does not reduce $^{35}Cl^-$ NMR linewidths in the S_1 state indicates that binding of Cl^- in competition with NH_3 either does not occur in the dark or has a long residence lifetime. Since EPR data [7,8] indicate that Cl^- binding to PS II in competition with NH_3 does occur in the dark, we conclude that such binding is in slow exchange on the NMR timescale so that the residence lifetime in free solution is longer than 50 ms. Further, we conclude that the ^{35}Cl NMR line broadening observed is mainly due to adventitiously bound paramagnetic cations and to non-specific binding sites. This conclusion explains the variability of line broadening in PS II samples (see table 1); different PS II samples probably contain a variable amount of extraneous Mn^{2+}.

Following from our conclusion that Cl^- is in slow exchange in this system, we suggest that high resolution ^{35}Cl NMR is not useful in studying binding of Cl^- to the OEC in the S_1 state. This is in disagreement with the conclusions of Govindjee, Gutowsky and co-workers [12–15] but is consistent with the findings of Preston and Pace [16] working with mangrove PS II at high $[Cl^-]$. It should be noted that under other circumstances, exchange at the Cl^--binding site might be fast enough to contribute to $^{35}Cl^-$ line broadening. By analogy with the faster exchange kinetics of Ca^{2+} when the 17 and 23 kDa extrinsic polypeptides are removed [31] it may be that these polypeptides slow the exchange of Cl^- in the dark and that after their removal one may observe NH_3-sensitive $^{35}Cl^-$ line broadening.

Acknowledgements: This work was supported by NSF grant DBM861630 and NIH grant GM32715. We wish to thank Professor R.G. Shulman for helpful discussion of the experiments.

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