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Exploring the Calcium-Binding Site in Photosystem II Membranes by Solid-State ^{113}Cd NMR†

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ABSTRACT: Calcium (Ca^{2+}) is an essential cofactor for photosynthetic oxygen evolution. Although the involvement of Ca^{2+} at the oxidizing side of photosystem II of plants has been known for a long time, its ligand interactions and mode of action have remained unclear. In the study presented here, ^{113}Cd magic-angle spinning solid-state NMR spectroscopy is used to probe the Ca^{2+}-binding site in the water-oxidizing complex of $^{113}Cd^{2+}$-substituted PS2. A single NMR signal 142 ppm downfield from $Cd(ClO_4)_2 \cdot 2H_2O$ was recorded from Cd^{2+} present at the Ca^{2+}-binding site. The anisotropy of the signal is small, as indicated by the absence of spinning side bands. The signal intensity is at its maximum at a temperature of −60 °C. The line width of the proton signal in a WISE (wide-line separation) two-dimensional $^{1}H-^{113}Cd$ NMR experiment demonstrates that the signal arises from Cd^{2+} in a solid and magnetically undisturbed environment. The chemical shift, the small anisotropy, and the narrow line of the ^{113}Cd NMR signal provide convincing evidence for a 6-fold coordination, which is achieved partially by oxygen and partially by nitrogen or chlorine atoms in otherwise a symmetric octahedral environment. The absence of a ^{113}Cd signal below −70 °C suggests that the Ca^{2+}-binding site is close enough to the tetramanganese cluster to be affected by its electron spin state. To our knowledge, this is the first report for the application of solid-state NMR in the study of the membrane-bound PS2 protein complex.

Calcium (Ca^{2+})[1] is an important cofactor for photosynthetic oxygen evolution, since Ca^{2+} depletion abolishes the O_2-evolving capability of photosystem II of plants (PS2). This has raised considerable interest in determining the structural and functional characteristics of the Ca^{2+}-binding site in PS2 (*1*−*5*). Water oxidation presumably takes place at a cluster of four manganese ions (*5*). Both the atomic structures of the tetra-Mn and of the Ca^{2+} sites of the photosynthetic water-oxidizing complex (WOC) are largely unknown. FTIR studies suggest that Mn and Ca^{2+} are bridged via a carboxylate group (*6*) to the tetra-Mn cluster, and Mn EXAFS data indicate that Ca^{2+} binds about 3.7 Å from a Mn (*7*, *8*). On the basis of the Mn^{2+} EPR signal, it has been proposed that Ca^{2+} organizes the binding site for Mn ions in the protein during the light-induced self-assembly of the tetra-Mn cluster (*9*). In particular, the ligands of Ca^{2+} and the geometry of its coordination sphere have not yet been identified.

One reason for the lack of understanding of the properties of the Ca^{2+}-binding site in PS2 is that spectroscopy of Ca^{2+}

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[1] Abbreviations: Ca^{2+}, calcium; Cd^{2+}, cadmium; CP, cross polarization; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; EPR, electron paramagnetic resonance; Mn, manganese; MAS, magic-angle spinning; NMR, nuclear magnetic resonance; PS2, photosystem II; WOC, water-oxidizing complex; WISE, wide-line separation.

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in its binding sites is difficult. The closed-shell d-electron configuration in Ca^{2+} does not provide any clearly definable transition that could be utilized to monitor with UV−visible spectroscopy the status of ligation. In addition, Ca^{2+} does not have unpaired electrons, and hence, one cannot perform EPR experiments. Finally, ^{43}Ca is not suitable for NMR because of its low gyromagnetic ratio γ and a quadrupole moment associated with the nuclear spin of $^7/_2$ (*10*).

A viable alternative is to replace Ca^{2+} with cadmium (Cd^{2+}) in the PS2 complex. Cd^{2+} has the same charge as Ca^{2+} and an ionic radius similar to that of Ca^{2+}, and it has two established $I = {}^1/_2$ NMR isotopes, ^{111}Cd and ^{113}Cd (*10*−*12*). In recent years, ^{113}Cd NMR has been recognized as a powerful tool for the exploration of the binding properties of metalloproteins, in which the cation can be substituted with Cd^{2+} (reviewed in refs *10* and *12*). The ^{113}Cd chemical shift and its anisotropy are very sensitive to the nature, number, and geometric arrangement of the ligands within the coordination sphere. Within its large chemical shift range of approximately 1000 ppm, oxygen is the most shielding and sulfur the most deshielding biologically relevant ligand (*10*). The line width and shape of the NMR signal provide additional information about the ligation symmetry and dynamics of the observed atom.

Here we provide the first results in probing the Ca^{2+}-binding sites in the photosynthetic PS2 protein complex by selective replacement of Ca^{2+} by $^{113}Cd^{2+}$ in the WOC of PS2 and solid-state ^{113}Cd NMR spectroscopy on frozen PS2 membranes. It will be shown that $^{113}Cd^{2+}$-substituted PS2 is a good system for probing the Ca^{2+}-binding site with ^{113}Cd cross-polarization (CP) magic-angle spinning (MAS) NMR. Solid-state CP/MAS NMR is a rapidly growing technique in the study of membrane proteins (*13*) and has already been applied to some important membrane proteins, including bacterial photosynthetic reaction centers (*14*−*16*). Our study is the first CP/MAS NMR investigation of PS2 membranes, opening a new observation window on the structure and function of the WOC.

MATERIALS AND METHODS

Preparation of $^{113}Cd^{2+}$-Substituted PS2 Membranes. PS2 membranes were prepared from spinach as described by Berthold et al. (*17*) with some modifications (*1*). Depletion of Ca^{2+} and the 17 and 23 kDa polypeptides was carried out by incubating starch-free PS2 membranes with SMN buffer [0.4 M sucrose/50 mM MES (pH 6.0) containing 2 M NaCl and 1 mM EGTA] with slow stirring for 30 min on ice in the dark, followed by exposure to daylight for 10 min. After centrifugation at 35000*g* for 25 min, the membranes were washed with SMN buffer (SM containing 10 mM NaCl) and repelleted. This preparation is termed salt-washed PS2 membranes. Ca^{2+} and $^{113}Cd^{2+}$ substitution was carried out by incubating salt-washed PS2 membranes with SMN containing either $CaCl_2$ or 99% enriched $^{113}CdCl_2$ at 5 mM (Cambridge Isotope Laboratories, Cambridge, MA) for 1 h in the dark, on ice with constant stirring. After centrifugation, the pellets were washed twice with SMN by resuspending the pellets in SMN and recentrifugation at 35000*g* for 30 min. Finally, pellets were resuspended in SMN. For the handling of Ca^{2+}-depleted samples, all utensils and containers were treated with 2 N nitric acid and all buffers were treated with Chelex 100. In one control experiment, PS2 membranes containing Ca^{2+} as well as the 17 and 23 kDa polypeptides were incubated with 5 mM $^{113}CdCl_2$ for 1 h in the dark, on ice with constant stirring. After centrifugation, the sample was washed twice with SMN buffer as described above. In another control experiment, $^{113}Cd^{2+}$-substituted PS2 membranes were resuspended in SMN buffer containing 50 mM $CaCl_2$ for 15 min to allow Cd^{2+} to be displaced by Ca^{2+}. After centrifugation, the sample was washed twice with SMN buffer as described above. The chlorophyll concentration in all samples was determined using the method of Arnon (*18*).

NMR Spectroscopy. One-dimensional ^{113}Cd CP/MAS NMR experiments were performed at low temperatures using a Chemagnetics CMX-400 spectrometer (Otsuka Electronics, Fort Collins, CO) equipped with a double-resonance 4 mm MAS probe operating at 88.8 MHz for ^{113}Cd. Twenty milligrams of PS2 membranes was placed inside a 4 mm rotor, and spectra were collected at temperatures of −20, −40, −60, −70, and −80 °C. The rate of spinning around the magic angle was kept at 9 kHz. Typically, about 25 000 scans were collected for every one-dimensional experiment with an acquisition time of 8.2 ms and a recycle time of 3 s. The 90° pulse length for ^1H was 4 μs, and a cross-polarization time of 2.5 ms was used. The ^{113}Cd signal has been enhanced by variable-amplitude cross polarization (VACP). During acquisition, protons were continuous-wave decoupled to remove the heteronuclear broadening efficiently. The chemical shifts are calibrated using solid $Cd(ClO_4)_2 \cdot 2H_2O$ as an external reference.

The two-dimensional (2D) $^{113}Cd-^1H$ WISE (wideline separation) NMR spectrum (*19*) was recorded at −60 °C for ^{113}Cd-substituted PS2 membranes with a DSX-300 spectrometer (Bruker, Karlsruhe, Germany) using a double-resonance magic-angle spinning probe operating at 66.6 MHz. The 90° pulse width for ^1H and ^{113}Cd pulses was 3.5 μs. The cross-polarization time was 2.5 ms. The data matrix had a size of 1024 complex data points in the t_2 (^{113}Cd) dimension. The spectral widths in t_1 and t_2 were 100 kHz and 1 MHz, respectively, corresponding to dwell times of 5 and 0.5 μs. The 64 experiments with 3000 scans each were collected with a repetition time of 1 s. The chemical shifts are referenced with respect to solid $Cd(ClO_4)_2 \cdot 2H_2O$.

RESULTS

Figure 1A shows the ^{113}Cd-CP/MAS NMR spectrum of dark-adapted ^{113}Cd-substituted PS2 membranes. A single rather narrow center band signal arises, which is almost symmetric. The intensity of the spinning side bands is negligibly small. Figure 1B shows that the signal shape can be fitted by a single Lorentz component with a maximum at 142 ppm and a line width of 8500 Hz. In one control experiment, using Ca^{2+}-containing, $^{113}Cd^{2+}$-treated PS2 membranes, a ^{113}Cd NMR signal was not observed (Figure 1D). These results show that the signal around 142 ppm in Ca^{2+}-depleted and Cd^{2+}-substituted PS2 membranes (Figure 1A) solely arises from the Cd^{2+} present in the Ca^{2+}-binding sites, and that Cd^{2+} does not bind to the PS2 membranes at any other sites when the Ca^{2+}-binding sites are already occupied with Ca^{2+}. In another control experiment, Cd^{2+}-substituted PS2 membranes were treated with Ca^{2+} to displace Cd^{2+}. These Ca^{2+}-reconstituted membranes restored

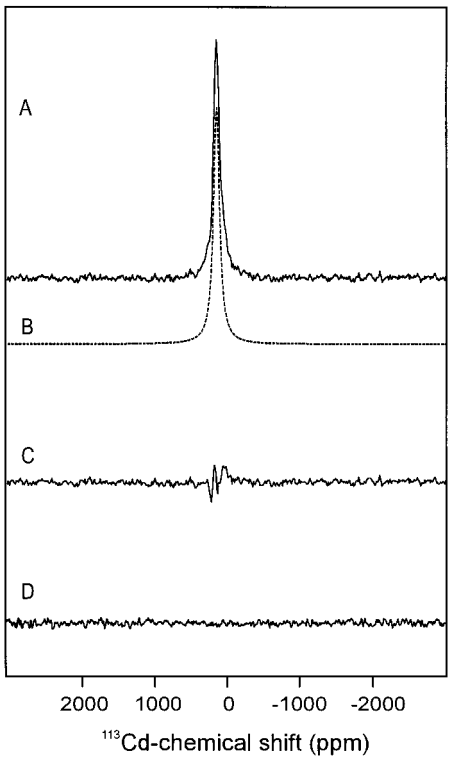

FIGURE 1: (A) Cross-polarization MAS ^{113}Cd NMR spectrum of $^{113}Cd^{2+}$-substituted PS2 membranes. (B) Least-squares fit of the spectrum with a Lorentz line. (C) Residue of spectrum A minus spectrum B. (D) Cross-polarization MAS ^{113}Cd NMR spectrum of Ca^{2+}-containing, $^{113}Cd^{2+}$-treated PS2 membranes.

up to 70% of the oxygen-evolving activity of normal PS2 membranes (*26*). No ^{113}Cd NMR signal was detected in these PS2 membranes.

The intensity of the ^{113}Cd signal at 142 ppm in ^{113}Cd-substituted PS2 membranes strongly depends on the temperature (Figure 2), indicating changes of the chemical or magnetic environment of the $^{113}Cd^{2+}$ ion. The strongest signal is observed at −60 °C (Figure 2C). The intensity of the signal at −20 °C is about 15 times weaker (Figure 2A). It increases at −40 °C (Figure 2B). When the sample cools to −70 °C, the signal intensity also decreases (Figure 2D). At a temperature of −80 °C, the signal is lost (Figure 2E). This observation contrasts with the common signal characteristics of ^{13}C or ^{15}N CP/MAS signals of labels in proteins, which generally can be observed without any difficulty at temperatures below −100 °C. It indicates a temperature-induced change in the local protein environment of the Cd^{2+}. No significant temperature dependency of the chemical shift and the line width are observed. In the very limited temperature window of about 40 °C, the intensities of the paramagnetic shifts are expected to be smaller than the spectral resolution (*20*).

In solution-state NMR spectroscopy, 2D ^{1}H−^{113}Cd NMR methods have been successfully applied in exploring metal−ligand connectivities (*21*, *22*). This approach has been very helpful in elucidation of the number and type of coordinating ligands at metal centers in "zinc fingers" (*23*, *24*). 2D heteronuclear dipolar correlation spectroscopy in the solid state is achieved in a very straightforward manner by ^{1}H wide-line separation (WISE) (*19*). Figure 3 shows a 2D ^{1}H−^{113}Cd WISE spectrum from $^{113}Cd^{2+}$-substituted PS2. Again, only a single line at 142 ppm can be detected in the ^{113}Cd

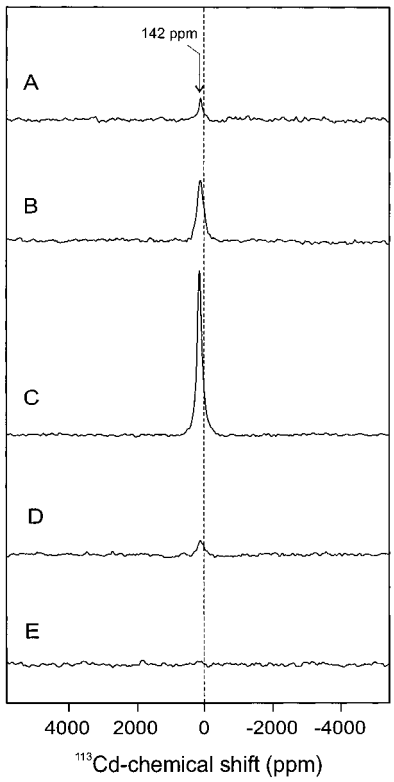

FIGURE 2: Cross-polarization MAS ^{113}Cd NMR spectrum of $^{113}Cd^{2+}$-substituted PS2 membranes as a function of temperature: (A) −20 °C (50 000 scans), (B) −40 °C (25 000 scans), (C) −60 °C (25 000 scans), (D) −70 °C (25 000 scans), and (E) −80 °C (10 000 scans).

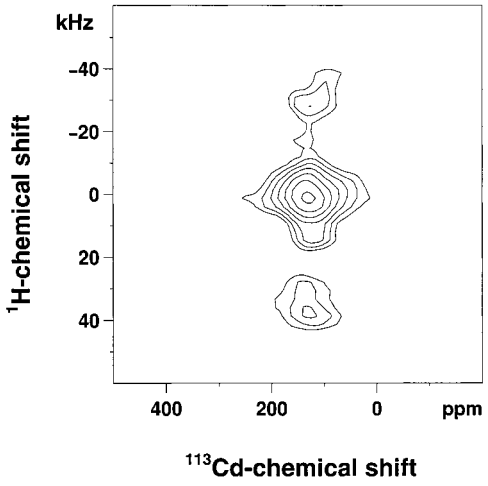

FIGURE 3: 2D ^{1}H−^{113}Cd WISE of $^{113}Cd^{2+}$-substituted PS2 membranes. The spectrum is plotted with eight contour levels increasing by a factor 1.1.

dimension. The half-line width Γ of 6 kHz of the dipolar broadened Gaussian proton signal is characteristic for MAS of protons in a frozen protein in a high field (*25*).

DISCUSSION

Number of Ca^{2+}-Binding Sites. Recently, we have reported the replacement of Ca^{2+} by Cd^{2+} in salt-washed PS2 membranes devoid of 17 and 23 kDa extrinsic polypeptides (*26*). These Cd^{2+}-substituted PS2 membranes were used in this study to characterize the Ca^{2+} site in PS2 at atomic level by using ^{113}Cd MAS NMR. Competition studies of Waggoner

and Yocum (*27*) have shown that Cd^{2+} can compete with Ca^{2+} for the binding site in the oxygen-evolving complex, since Cd^{2+} substitution disables the O_2-evolving activity of salt-washed PS2 membranes. The oxygen-evolving activity could be largely (>70%) restored after addition of Ca^{2+} to Cd^{2+}-substituted PS2 membranes (*26*). This shows that Cd^{2+} substitution and reconversion leaves the tetra-Mn complex and the Mn-stabilizing water-soluble 33 kDa protein essentially unaffected (*27*). The restoration of oxygen evolution via displacement of Cd^{2+} by Ca^{2+} confirms that Cd^{2+} had replaced Ca^{2+} at a functionally important binding site.

From the line width of the Gauss-shaped proton signal in the WISE 2D $^1H-^{113}Cd$ NMR experiment (Figure 3), there is no indication for magnetic broadening of the ^1H signal in the immediate environment of the Cd^{2+}. Also, the relatively narrow ^{113}Cd signal in Figure 1 suggests the absence of broadening by unpaired electrons in the vicinity of the Ca^{2+}-binding site. The occurrence of only a single Lorentz-shaped signal (Figure 1) is consistent with the presence of only one Ca^{2+}-binding site in PS2 (*28*). In both control experiments (Figure 1D), after Cd^{2+} treatment of Ca^{2+}-retaining PS2 membranes or after Ca^{2+} reconstitution of Cd^{2+}-substituted PS2 membranes, no ^{113}Cd NMR signal was detected. These results confirm that the ^{113}Cd signal in the Cd^{2+}-substituted PS2 membranes solely arises from the Cd^{2+} present at the Ca^{2+}-binding site.

Coordination Number and the Type of Ligands. The signal at 142 ppm is highly symmetric, as indicated by the Lorentz fit, with a rather narrow half-line width of 8.5 kHz. These, and the complete absence of spinning side bands, indicate a very symmetric charge density distribution at the metal with a symmetrical six- or eight-coordinate Cd^{2+} (*29*−*31*). A square-planar, tetrahedral, pyramidal, or heptagonal coordination geometry is unlikely. Four-coordinate spheres, even if oxygen-rich, are mostly too deshielding for a chemical shift value of less than 200 ppm. On the other hand, seven or eight coordination, which requires small ligands such as oxygen, causes chemical shifts from −50 to −200 ppm (*10*, *12*). Calcium exhibits a general preference for coordination numbers six (ionic radius of 100 pm) and eight (radius of 112 pm); Cd^{2+} prefers coordination numbers four (78 pm) and six (95 pm), while the chemical shift suggests five or six coordination. The very high efficiency of exchange of Ca^{2+} by Cd^{2+}, as demonstrated previously (*26*), indicates that the coordination number, which is probably six, remains.

The chemical shift of the ^{113}Cd signal is also slightly outside the range expected for a coordination sphere of six oxygen atoms, around 0 ppm (*10*). Therefore, it is reasonable to assume that Cd^{2+} is also ligated by one or two nitrogen or chlorine atoms. Ligation by sulfur atoms would shift the response to higher frequencies (see below) and is therefore very unlikely. This seems to rule out the possibility that the inhibition of oxygen evolution results from specific interactions of the heavy metal with protein −SH groups or the disulfide bridge in the 33 kDa protein [which was already shown not to be required (*32*)]. The chemical shifts of ca. 140 ppm, as we observed in $^{113}Cd^{2+}$-substituted PS2 membranes, were also reported in other Cd^{2+}-substituted enzymes with a mixed oxygen/nitrogen ligation sphere (for a review, see ref *12*). For example, the Cd in the Zn binding site of alkaline phosphatase, formed by three nitrogen and two oxygen atoms (*33*), has a chemical shift of ca. 140 ppm (*34*, *35*). Titration with chlorine causes a shift to ca. 160 ppm (*36*). In insulin hexamers, aggregated upon addition of zinc, the Zn binding site is six coordinated by three histidine nitrogens and three water oxygen atoms. Cd^{2+}-substituted samples exhibit a ^{113}Cd chemical shift at 165 ppm (*37*). The Zn binding site of carboxyl peptidase, where Zn is five-coordinated by two histidine nitrogens, two glutamic acid oxygens, and one weakly bound water oxygen (*38*), exhibits a single signal at 120 ppm, which shifts to around 340 ppm upon binding of a sulfur donor (*39*). The chemical shift of ^{113}Cd in $Na_2[Cd(EDTA)]$ solid, where two nitrogen and four oxygen atoms provide an octahedral coordination sphere, is at 122 ppm (*40*). Hence, in line with the chemical shift, the absence of anisotropy, and the high substitution efficiency, we assign the signal to a symmetric six-coordinate sphere of oxygen and nitrogen or chlorine.

Location of the Ca^{2+}-Binding Site. The strong dependence of the intensity of the signal on the temperature is remarkable (Figure 2). The strongest signal is observed at −60 °C. A possible cause for the weak signal at −20 °C is the lack of cross polarization in not completely frozen samples. Since the signal has not reached its full intensity at −40 °C, when the protein bulk phase is very rigid, dynamic surface exchange processes may also play a role. Therefore, the observed mobility at the Ca^{2+}-binding site may be interpreted in terms of a very accessible position.

The question of why the signal is suppressed at −80 °C remains. This intensity change of the ^{113}Cd NMR signal could be interpreted in terms of a change of the magnetic environment. The NMR sample in our experiments contained a tetra-Mn cluster in the S_1 oxidation state, to which the system relaxes in the dark. In the S_1 state, the tetra-Mn cluster presumably forms a Mn_4^{14+} cluster with a d^{14} electron configuration (*41*). In an octahedric crystal field, a high-spin system can be assumed. In $Mn^{4+}Mn^{4+}$ (d^6) and Mn^{3+}-Mn^{3+} (d^8) dimers, electrons are localized and form strongly antiferromagnetically coupled $S = 0$ ground states (*42*). A d^7 dimer is expected to form mixed-valence $Mn^{3.5+}Mn^{3.5+}$ systems with a delocalized ($S = {}^1/_2$) electron (*42*). The d^{14} tetramer can carry either an $S = 1$ net spin or an $S = 0$ total electron spin. The $S = 1$ ligand is realized in a ferromagnetically coupled paramagnetic $Mn_2(d^{7\uparrow})Mn_2(d^{7\uparrow})$ configuration, while the $S = 0$ spin requires either an antiferromagnetically coupled paramagnetic $Mn_2(d^{7\uparrow})Mn_2(d^{7\downarrow})$ configuration or a diamagnetic $Mn_2(d^{8\downarrow\uparrow})Mn_2(d^6)$ system. Magnetic susceptibility measurements on O_2-evolving PS2 preparations have indicated an effective magnetic moment μ_{eff} of 6−10 μ_B for the Mn cluster (*43*). As $\mu_{eff} = 4S(S + 1)$, this leads to an $S = 1$ effective spin. With a paramagnetic $S = 1$ environment, the loss of the ^{113}Cd NMR signal intensity below −70 °C can be attributed to pronounced line broadening or to a breakdown of cross polarization by the paramagnetism of the tetra-Mn cluster. There is no experimental hint for paramagnetic broadening. On the other hand, the cross-polarization efficiency can decrease due to shortening of the $T_{1\rho}$ or a paramagnetic shift of the proton signal out of the window for Hartmann−Hahn matching (*25*). In general, the relaxation rate of *J*-coupled paramagnetic metal centers is determined by the fastest among the metal ions (*44*). For d^3 and d^4 systems, very short $T_{1\rho}$ values are indeed observed (*44*). This confirms that the Ca^{2+}-binding site is in the vicinity of the tetra-Mn complex.

With parallel mode EPR, a broad "$g = 4.8$" signal has been detected for the S_1 state (*45*, *46*). It was first detected at 4 K, and could be observed upon warming to 200 K in the dark. It has been assigned to an excited state of an $S = 1$ spin with a separation from the $S = 0$ ground state of about 2.5 K. Recently, also an EPR "$g = 12$ multiline" signal with at least 18 hyperfine lines for the S_1 state at 3.8 K in samples without the 17 and 23 kDa subunits has been reported (*47*). Hence, there is EPR evidence for paramagnetism on the tetra-Mn cluster in the S_1 state below −70 °C.

When all the observations are taken together, a picture of a six-coordinate Ca^{2+}-binding site with a symmetric mixed oxygen and nitrogen and/or chlorine coordination sphere, located close to the tetra-Mn cluster, emerges. Our study demonstrates that Cd^{2+} in the Ca^{2+}-binding site of PS2 can serve as a "spin spy" of the magnetic state of the tetra-Mn cluster, and it shows that ^{113}Cd MAS NMR spectroscopy can be fruitfully applied in obtaining structural and functional information about the PS2 complex. Further studies, probing the paramagnetic S_1-state properties below −70 °C as well as other states of the S cycle by ^{113}Cd CP/MAS NMR, are currently undertaken.

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