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Triple resonance EPR spectroscopy determines the Mn^{2+} coordination to ATP

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Abstract

Mn^{2+} often serves as a paramagnetic substitute to Mg^{2+} , providing means for exploring the close environment of Mg^{2+} in many biological systems where it serves as an essential co-factor. This applies to proteins with ATPase activity, where the ATP hydrolysis requires the binding of Mg^{2+} -ATP to the ATPase active site. In this context, it is important to distinguish between the Mn^{2+} coordination mode with free ATP in solution as compared to the protein bound case. In this work, we explore the Mn^{2+} complexes with ATP, the non-hydrolysable ATP analog, AMPPNP, and ADP free in solution. Using W-band ^{31}P electron-nuclear double resonance (ENDOR) we obtained information about the coordination to the phosphates, whereas from electron-electron double resonance (ELDOR) – detected NMR (EDNMR) we determined the coordination to an adenosine nitrogen. The coordination to these ligands has been reported earlier, but whether the nitrogen and phosphate coordination is within the same nucleotide molecules or different ones is still under debate. By applying the correlation technique, THYCOS (triple hyperfine correlation spectroscopy), and measuring ^{15}N - ^{31}P correlations we establish that in Mn-ATP in solution both phosphates and a nitrogen are coordinated to the Mn^{2+} ion. We also carried out DFT calculations to substantiate this finding. In addition, we expanded the understanding of the THYCOS experiment by comparing it to 2D-EDNMR for ^{55}Mn - ^{31}P correlation experiments and through simulations of THYCOS and 2D-EDNMR spectra with ^{15}N - ^{31}P correlations.

Graphical abstract

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Keywords

Mn²⁺ ion; ATP; high field EPR; ENDOR; ELDOR detected NMR; THYCOS; Mn²⁺-ATP

Introduction

Adenosine triphosphate (ATP) hydrolysis, carried out by a great variety of proteins having ATPase activity, requires the presence of Mg²⁺ ions. Many crystal structures of the relevant proteins show that the non-hydrolysable ATP analogs, AMPPNP and ATP-γS, and ADP, bind to the ATPase active site in the form of the Mg²⁺ complex [1–6], where the Mg²⁺ is coordinated both to the AMPPNP molecule and to one or more protein residues. In the case of the free Mg²⁺/ATP complex some studies report that all three phosphates are coordinated [7, 8], while others claim that only the γ- and β- phosphates can be coordinated to the same metal ion [9, 10]. There are also reports that in solution both complexes with 2 and 3 bound phosphates co-exist [11], while others report that two metal ions bind to the same nucleotide molecule [12–14]. Coordination of metal ion to the adenine moiety is possible as well [15, 16]

A close look at the Mg²⁺/ATP binding site in proteins in solution can be achieved by substituting the diamagnetic Mg²⁺ with paramagnetic Mn²⁺ for EPR observation [17], [18, 19]. There have been numerous reports that this substitution retains the ATPase activity [20–22]. A prerequisite of such studies is the investigation of the structure of the Mn²⁺ complexes with ATP analogs and ADP, free in solution as a reference, because it can coexist with the protein-bound complex. Therefore, structures of the metal-nucleotide complexes in solutions have been extensively studied [11, 16, 23]. It has been shown with optical spectroscopy and X-ray diffraction that when ATP concentration is low a 1:1 Mn-ATP is formed, whereas in excess of ATP a Mn²⁺-(ATP)₂ complex is generated [12, 24]. Some studies report that under excess ATP conditions, the Mn²⁺ ion is bound to phosphates of one ATP molecule and the nitrogen of the adenine ring of a second one, which is stacked next to the first molecule as in the crystal [14, 25, 26]. A similar situation arises in buffer with excess salt concentrations [27]. Two main models of Mn²⁺/Mg²⁺ interaction with adenine moiety have been suggested: either the metal ion coordinates three phosphates of the ATP and interacts with adenine through a hydrogen bond, or it coordinates β and γ phosphates and the N₇ of the adenine directly, while the α phosphate shares a hydrogen bond with the

metal ion [28]. Fig. 1 presents a number of possible coordination modes reported in the literature; in principle, an equilibrium between several forms can exist.

Recently it has been shown that W-band ELDOR-detected NMR (EDNMR) [29–32] is a particularly useful method to probe the coordination shell of Mn^{2+} in general [32–36] and to detect $^{14}\text{N}/^{15}\text{N}$ coordination to Mn^{2+} complexes with ATP, AMPPNP and ADP (Mn-ATP, Mn-AMPPNP, Mn-ADP) free in solutions and bound to proteins at the ATPase site [37]. This method is also very useful to detect the ^{31}P signals from the nucleotide phosphates, but the spectra have lower resolution than those obtained with ENDOR (electron-nuclear double resonance) and therefore, it cannot unambiguously distinguish the different phosphates, while ENDOR can. Accordingly, the combined application of ENDOR and EDNMR is preferred [37]. While EDNMR and ENDOR are highly efficient in detecting the presence of ^{14}N and ^{31}P in the Mn^{2+} coordination shell, they cannot determine if the two types of nuclei are coupled to the same Mn^{2+} ion. Namely, it cannot distinguish between coordination mode (a) or a superposition of (b) and (c), shown in Fig. 1. Here we show that by the application of the triple hyperfine correlation experiment, THYCOS [38], which correlates EDNMR and ENDOR spectra, it is possible to determine whether the coupled nitrogen and phosphates belong to the same molecule. This was achieved by applying THYCOS on Mn-ATP, fully enriched with ^{15}N , showing that coordination mode (a) (Fig. 1), where the Mn^{2+} is coordinated to phosphates and one nitrogen, most probably the N_7 , is present in solution. Similar information, though with lower resolution can be obtained by the triple resonance experiment, two-dimensional (2D) EDNMR [39]. We present simulations of the THYCOS and 2D-EDNMR spectra that substantiate this assignment. In addition, we also carried out DFT (density functional theory) calculations that show that Mn^{2+} coordination to two or three phosphates and N_7 is stable and the calculated ^{14}N hyperfine couplings derived from such an optimized structure are in reasonable agreement with the experimental values.

Experimental

Sample preparation

Tri-sodium phosphate (Na_3PO_4), Adenosine triphosphate (ATP), ^{15}N labeled adenosine triphosphate $^{15}\text{N}_5$ -ATP, Adenosine diphosphate (ADP), and Adenosine 5'-(β,γ -imido)triphosphate (AMPPNP) were purchased from Sigma-Aldrich and used as is. As the nucleotides are prone to degradation in solutions, the stock solutions were monitored using the UV-Vis spectrum ($\epsilon[280\text{nm}]=15400\text{M}^{-1}\text{cm}^{-1}$) [40]. The nucleotides were dissolved in HEPES 20mM NaCl 50uM, 30% glycerol pH=6.8 (the buffer was chosen to match one of the most common buffers used in biochemical and biophysical experiments) to a final concentration of 100mM. MnCl_2 was prepared separately in the same buffer at the concentration of 100mM. The final samples were prepared from stock solutions by volumetric dilution with buffer to the final concentrations 240 μM nucleotide, 200 μM MnCl_2 , 20mM HEPES, 50 μM NaCl, 30% glycerol. The samples of Mn-Pi were prepared with a similar procedure, but instead of the nucleotide trisodium phosphate (Na_3PO_4) was used. The final concentration of Mn-Pi sample was 20mM Na_3PO_4 , 200 μM MnCl_2 , 20mM HEPES, 50 μM NaCl, 30% glycerol, pH = 7.

EPR spectroscopic measurements

Spectroscopic measurements were performed on a home-built W-band (94.9 GHz, ~3.4 T) spectrometer[41, 42]. All measurements were done at a temperature of 10K. The magnetic field was fixed at the maximum of the second hyperfine component of Mn^{2+} ($I = 5/2$, ($|m_S, m_I\rangle = |-1/2, -3/2\rangle \rightarrow |+1/2, -3/2\rangle$) (Supplementary materials , SI, Figure S1).

ENDOR

The standard Davies ENDOR sequence[43] $t_\pi - T - t_{\text{RF}} - T - t_{\pi/2} - (\tau - t_\pi - \tau - \text{echo})_k$ complemented with CPMG detection[42, 44] was used (Fig. 2a). The echo intensity was recorded as a function of the radio frequency (RF) , swept from 50.7 to 66.7 MHz and a step of 50 KHz, using the random acquisition mode to minimize the effect of the insufficient nuclear relaxation induced artifacts, and also reduce baseline noise due to the heating[45]. The microwave (mw) pulse lengths were set to $t_{\pi/2} = 100$ ns, $t_\pi = 200$ ns, the RF pulse length (t_{RF}) was set to 50 μs and $\tau = 1\mu\text{s}$. Additional delays of 2 μs before and 8 μs after the RF pulse were added in order to avoid artifacts from ring-down effects. The full time domain transient with all the echoes was recorded for each RF frequency and the echoes were integrated post-measurement using an in-house Matlab script. The integration window and number of echoes integrated were chosen to produce the best signal to noise ratio (SNR) and were kept constant for all samples. The best SNR was found for 11 echoes integrated with 96 ns gate positioned symmetric to the maximum.

EDNMR and 2D-ELDOR

Standard ELDOR pulse sequence[29] $t_{\text{HTA},2} - T - t_{\pi/2,1} - (\tau - t_{\pi,1} - \tau - \text{echo})_k$ complemented with CPMG detection was used (Fig. 2b)[42] . The echo is generated at a frequency of $\nu_1 = 94.9\text{GHz}$ with the field set the same as in the ENDOR experiment. The frequency of the HTA pulse, ν_2 , is swept in several different ranges and the full echo signal was recorded as a transient in the time domain trace for each frequency. The detection echo sequence parameters were $t_{\pi/2,1} = 100$ ns, $t_{\pi,1} = 200\text{ns}$, $\tau = 1\mu\text{s}$. The delay T between the HTA pulse and echo generating sequence 2 μs and the shot repetition time was set to 1ms. The duration of the HTA pulse, $t_{\text{HTA},2}$ was optimized to achieve good balance between spectral resolution and SNR. The pulse length was chosen to be long enough to be highly selective and the microwave power was optimized to increase the ELDOR effect (Supplementary materials Figure S2). The full range EDNMR scans were recorded by sweeping ν_2 from 94.9 GHz to 95.05 GHz with a step of 100 KHz. For the ^{31}P region the ν_2 was swept from 94.95 GHz to 94.97 GHz in steps of 50 KHz, for the ^{14}N region the frequency was swept from 94.9 GHz to 94.925 GHz in steps of 40 KHz and for ^{15}N region the region was from 94.9 GHz to 94.930 GHz in steps of 40 KHz. As mentioned for the ENDOR measurements the transient signal was integrated using a Matlab script and the integration window was optimized to produce the best spectral resolution while keeping reasonable SNR[46]. The integration gate of 300 ns gave the best SNR and the number of echoes integrated was 6. The resulting EDNMR spectra were post-processed in order to remove the central hole by fitting a Lorentzian[46]. The EDNMR efficiency, i.e. the ELDOR effect, was calculated as the difference between the fitted central hole shape and the

experimented data, both being normalized to the intensity of the signal at 150 MHz (Supplementary materials Figure S3).

2D EDNMR, or double EDNMR experiments were performed according to the pulse sequence presented in Figure 2d[39] where the frequencies of the two HTA pulses $\nu_{\text{HTA1}} = \nu_2$ and $\nu_{\text{HTA2}} = \nu_3$ are swept. The frequency ν_2 was swept in the range 94.95 -94.97GHz with a 100 KHz step. In practice, the frequency of the third channel, ν_3 , was not swept continuously, but was rather set to several predefined spectral positions corresponding to the ^{15}N and ^{31}P spectral lines. In addition, it was set to a remote frequency for the purpose of baseline correction (SI, Figure S4). The baseline correction was performed in a slightly different way from the procedure reported earlier: instead of dividing the signal by the reference spectrum we subtracted the reference spectrum, recorded with ν_3 at 95.3GHz ($\nu_{\text{HTA2}} = 400$ MHz). We saw that in the spectral region of interest (ν_{HTA2} from 52 MHz to 58 MHz) the two approaches produce practically the same SNR. The same treatment was used for the simulated spectra. In the simulations the central blind spot is far from ideal and the division by the reference can sometimes introduce artifacts, which complicate the normalization of the signals. Therefore, we used subtraction.

THYCOS—The THYCOS experiment[38] was performed using the previously reported sequence complemented with CPMG detection (See Fig. 2c)[42]. The parameters of the pulses were the same as in the ENDOR and EDNMR experiments: $t_{\text{HTA}} = 100 \mu\text{s}$, $t_{\text{RF}} = 50 \mu\text{s}$, $t_{\pi/2,1} = 100$ ns, $t_{\pi,1} = 200$ ns, $\tau = 1 \mu\text{s}$. In order to avoid artifacts from ringdown effects the delays $T_1 = 1 \mu\text{s}$ and $T_2 = 5 \mu\text{s}$ were introduced. The transient echo signal was recorded as a function of the ν_{RF} for several ν_2 values. The RF was swept in range 50.7 MHz to 66.7 MHz in 50 KHz steps. The mw frequencies were chosen to match specific spectral lines of the forbidden transitions associated with the investigated nuclei. Integration was done with an integration window of 96 ns, and 9 echoes were used for the best SNR and a window of 300 ns and 6 echoes for the best spectral resolution. THYCOS spectra require no subtraction of a reference signal.

Simulations

The EDNMR and slices of 2D-EDNMR spectra were simulated according to the previously reported procedure where the HTA pulse saturates the forbidden transition[47]. The program has been adjusted to include several nuclei in the spin system. The simulation parameters are given in Table 1. To account for the orientational disorder of the sample all orientations of the hyperfine tensor with respect to the magnetic field were considered and summed. For simplicity, all tensors were taken as coinciding. In these simulations we assumed an effective electron spin of $S=1/2$, which is justified as the magnetic field was set to the central transitions of the Mn^{2+} , where the contributions of the other transitions to ENDOR and EDNMR spectra are negligible.

A simplistic calculation of THYCOS spectrum was performed using the same program, modified to replace one of the HTA pulses with an ideal, 180° inversion RF pulse. ν_{HTA} was set either to an NMR transition or far away from any NMR transition and the RF was swept in range 52-66MHz with a 50kHz step. The line widths were introduced by

convolution with normalized Gaussians. This, of course, does not account for partial inversion, but does consider the effects of relaxation and hyperfine parameters distributions.

DFT Calculations

Density functional theory (DFT) calculations were carried out using Gaussian 09 (Revision B.01)[48] and ORCA[49]. The former was used to optimize geometries using the B3LYP [48–51]/6-31+G(D,P)[50–53] hybrid density functional and basis-set and default options. The latter was used to obtain the hyperfine coupling values using the PBE0[54] functional and 6-31+G(D,P)[50–53] basis for carbon and oxygen atoms and EPR-III for phosphorus, nitrogens and hydrogens. These were combined with the tight SCF and Grid5 ORCA options. Normal mode analysis showed that the geometries were stationary points. There was no particular reason for using Gaussian over ORCA for the geometry optimization and we have not carried out a comparative study of the geometry optimization between G09 and ORCA. For the calculation of the hyperfine coupling we used ORCA because it yields better hyperfine coupling because of its more comprehensive treatment than Gaussian.

Results and Discussion

ENDOR and EDNMR

The Davies ^{31}P -ENDOR spectra of the Mn^{2+} complexes with ATP, AMPPNP and ADP are shown in Fig. 3. The spectra are similar to those reported earlier [19, 37, 55–57] and the main difference between the ADP complex and the ATP/AMPPNP complexes are the shoulders at $\pm 1\text{MHz}$, indicated by the arrows in Fig. 3. The spectra were simulated using EasySpin [58] assuming that the anisotropic hyperfine tensor, $\mathbf{T}$, can be described by the point-dipole approximation, keeping to a minimum the number of coupled nuclei and taking into account the Davies ENDOR suppression effect around the nuclear Larmor frequency [59]. The Mn-ADP spectrum could be simulated using only one type of coupled ^{31}P , as reported earlier [56], while for Mn-ATP and Mn-AMPPNP two different types of ^{31}P were required, with slightly different parameters for ATP and AMPPNP. The parameters employed in the simulations are given in Table 2 and the simulations are compared with the experiment in Fig. 3. The spectra could be well simulated considering only contributions from the $m_s = \pm 1/2$ electron spin manifolds. While these simulations indicated two coordinated phosphates for ATP and AMPPNP for ADP it could be one, or two phosphates with the same couplings. Based on these simulations the shoulders at $\pm 1\text{MHz}$ can be assigned to the γ phosphate under the assumption that the coupling of the β -phosphate is the same in ADP and ATP, which is not necessarily true, as suggested by the DFT results [57].

^{31}P hyperfine coupling parameters of the Mn-nucleotides complexes have been reported earlier[19, 56, 57] giving conflicting results regarding the number of phosphates coordinated by Mn^{2+} and their couplings. While in one study two different ^{31}P nuclei were used to simulate Mn-ATP and Mn-AMPPNP[56] in another study only one type was used together with the application of a significant distribution over the hyperfine coupling[19] but intensity consideration lead to the conclusion that two equivalent ^{31}P nuclei were coordinated. Recently simulations based on DFT calculations were reported and the corresponding ^{31}P couplings are listed in Table 1 as well[57]. The DFT calculations showed that anisotropic

hyperfine interaction is not axial and therefore the point-dipole approximation does not fully apply. It also predicted that the Mn^{2+} ion coordinates all three ATP phosphates and the two phosphates of the ADP molecule. Moreover, the shoulders at $\pm 1\text{MHz}$ are assigned to $\beta\text{-}^{31}\text{P}$ and not to $\gamma\text{-}^{31}\text{P}$ and upon hydrolysis the coupling of the $\beta\text{-}^{31}\text{P}$ changes significantly.

We provide additional support for the number of coordinated phosphates from the EDNMR spectra of Mn-ADP, Mn-AMPPNP, Mn-ATP. Figure 4a shows the full range EDNMR spectra and Fig.4b focuses on the ^{31}P region. It has been shown earlier that the EDNMR is particularly sensitive to the number of nuclei coordinated [32]. The EDNMR efficiency of the ^{31}P doublets was shown to be proportional to the number of the phosphates coordinated to the Mn^{2+} [37], provided that they have similar anisotropic hyperfine couplings. In case that Mn^{2+} is coordinated to all the phosphates of ATP and ADP, the intensity ratio of the corresponding spectral lines is expected to be 3:2 ($I_{31\text{P}}(\text{ATP})/I_{31\text{P}}(\text{ADP})$). The intensity ratio between of the ^{31}P peaks in Mn-ATP and Mn-ADP ($I_{31\text{P}}(\text{ATP})/I_{31\text{P}}(\text{ADP})$) was : 1.64:1 (Fig 4b)). Taking into account the differences in the dissociation constants of Mn-ATP and Mn-ADP ($K_d(\text{ATP}) = 3 \cdot 10^{-6}\text{M}$, $K_d(\text{ADP}) = 1 \cdot 10^{-5}\text{M}$ [60]) the ratio between EDNMR signals of ATP to ADP is corrected to 1.49:1. This number is close to 3:2 and supports the coordination of the Mn^{2+} to all phosphates for ATP and ADP. The $I_{31\text{P}}(\text{AMPPNP})/I_{31\text{P}}(\text{ATP})$ ratio of 1.15:1 changes to 1.04:1 after correcting for the different Mn^{2+} binding affinities of AMPPNP and ATP [6] which means that the Mn^{2+} binding mode is practically the same for these two complexes. In these calculations, we took into account the room temperature K_d assuming that the freezing by insertion into liquid nitrogen is rapid enough and does not allow for re-equilibration. Taking K_d values determined at 1°C would reduce the corrected $I_{31\text{P}}(\text{ATP})/I_{31\text{P}}(\text{ADP})$ ratio below 1.49:1.

The EDNMR spectra in the ^{14}N region are given in Fig. 4c. All samples show a broad signal at the ^{14}N Larmor frequency, corresponding to unresolved single quantum (sq) transitions, and two weak peaks corresponding to the double quantum (dq) transitions from which a coupling of 2.35MHz can be derived [57]. The intensity the sq peak for AMPNMP is stronger than for the other samples and the lineshape is slightly different, probably owing to the presence of the nitrogen in the PNP bridge. The relative intensity the dq lines are also weaker. Sq transitions with resolved hyperfine couplings was obtained using ^{15}N enriched ATP ($^{15}\text{N}_5\text{ATP}$), where a clear doublet with a splitting of 4.2 MHz is observed and assigned to the coordinated N_7 nitrogen. Taking into account the different γ values of ^{14}N and ^{15}N , this should correspond to a hyperfine coupling of 3 MHz, somewhat larger than the one determined from the ^{14}N double quantum lines. We attribute this discrepancy to the simple analysis ignoring anisotropic contributions to the splitting. The peak at the ^{15}N Larmor frequency is assigned to the other weakly coupled nuclei ^{15}N in the ATP molecule. These results are similar to those reported earlier[37]. The substantially larger line of ^{23}Na in the EDNMR spectrum of Mn- $^{15}\text{N}_5\text{ATP}$ is due to the composition of buffer: the $^{15}\text{N}_5\text{ATP}$ was purchased as a solution of Tris buffer and NaCl.

THYCOS and double-EDNMR

The EDNMR results provide evidence that a nitrogen is coordinated to the Mn^{2+} , but they do not allow distinguishing the cases where Mn^{2+} is coordinated to both the phosphates and

nitrogen (Fig. 1A), or that two types of complexes co-exist in the solution, one with phosphates bound and one with nitrogen bound (Fig. 1b,c). It is also possible that all three above mentioned configurations are present in solution. To resolve this, we have applied the THYCOS experiment, which provides correlations between nuclei that are coupled to the same Mn^{2+} ion.

Before carrying out THYCOS experiments correlating ^{15}N - ^{31}P and ^{14}N - ^{31}P , which suffer from low resolution in the $^{14}/^{15}\text{N}$ spectral region, we carried out ^{55}Mn - ^{31}P THYCOS, where the ^{55}Mn signals, corresponding to the $m_S=\pm 1/2$ manifolds appear at $\nu=94.5$ MHz and 172 MHz (Fig. 5a and b). These lines are well separated from each other and from other spectral lines, allowing addressing them with the HTA pulse without concerns for selectivity or overlap with the central hole and therefore they can be used as reference spectra. The mechanism of the THYCOS experiment is given in the SI, Fig. S5. The THYCOS spectra presented in Fig. 5b, exhibit, as expected, only one of the ^{31}P components as positive. The other component appears as a weak negative signal, although a zero signal is expected. For comparison we have also carried out the Double-EDNMR experiment, which is in principle similar to THYCOS (see Fig. 2) just the RF pulse is replaced with a second HTA pulse[38, 39]. In this experiment, we have set the first HTA pulse to one of the ^{55}Mn lines and swept the second HTA pulse. The results, presented in Fig. 5c are similar to those of the THYCOS experiments, except for the relative intensities of the positive and negative peaks. The negative lines may result from polarization transfer due to either electron-nuclear or nuclear-nuclear cross relaxation, which involves flips of both nuclei as described in Fig. S5 in the SI. This can occur within the long HTA pulse. As both experiments provides correlations between NMR transitions belonging to different electron spin manifolds (see Fig. S5), we conclude from this experiment that the hyperfine couplings of the ^{55}Mn and ^{31}P nuclei have opposite signs. Earlier experimental results reported the hyperfine coupling of $^{55}\text{Mn(II)}$ to be negative[61] and therefore we conclude that the ^{31}P coupling is positive.

Next we proceeded to carry out ^{15}N - ^{31}P correlation experiments on $\text{Mn-}^{15}\text{N}_5\text{ATP}$, where the two ^{15}N hyperfine components are resolved and we set the frequency of the HTA pulse to the high frequency component at $\nu=16.5$ MHz and the RF was scanned in the ^{31}P region. The THYCOS spectrum in Fig. 6 shows only the low frequency peak of the ^{31}P doublet. Considering that the gyromagnetic ratio of ^{15}N is negative, this reveals that the ^{15}N and ^{31}P hyperfine couplings have opposite signs and because the ^{31}P hyperfine coupling was found to be positive, the ^{15}N coupling has to be negative. Importantly, these results indicate that the ^{15}N and the ^{31}P are in the same molecule, namely both the N_7 and phosphates are coordinated to the same Mn^{2+} . Measurements carried out with the HTA pulse set to the ^{15}N low hyperfine frequency component and to the ^{15}N Larmor frequency gave poor results because they are affected to a large extent by the overlapping central hole (see Fig. S6).

We also carried out THYCOS experiments on the natural abundance complexes (see Fig. 6). In these experiments the HTA pulse is set to the ^{14}N Larmor frequency, ($\nu_{\text{HTA}}=10$ MHz) where the ^{14}N hyperfine components are not resolved and therefore the HTA pulse is not selective. In this case we expect to see both ^{31}P double components as positive signals (see SI). For the ADP complex we could not detect any THYCOS signal. In contrast, for AMPPNP and ATP the THYCOS spectra reveal both components of the ^{31}P doublet upon

excitation of the ^{14}N line, but the spectra consist of a superposition of positive and negative signals in different ratios. The absence of a THYCOS effect for Mn-ADP suggests that the N_7 and the phosphate are not coordinated to the same Mn^{2+} , but rather the solution contains complexes where the Mn^{2+} is coordinated to either the nitrogen or the phosphate. Considering the superposition of negative and positive signals for observed for ATP and AMPPNP, this suggested assignment need to be further established using ^{15}N -ADP.

To understand the origin of the negative signals appearing in the ^{14}N - ^{31}P THYCOS we used a reference sample, Mn^{2+} - P_i (Na_3PO_4), which has a ^{31}P ENDOR spectrum very similar to that of Mn-ATP (see Fig. S8a) and has no coordinated nitrogens[57]. To resolve potential contributions to the THYCOS spectrum from the HTA pulse affecting the allowed transitions we carried out measurements with the HTA frequency set to $\nu_{\text{HTA}}=5, 10$ and 16 MHz, as shown in Fig. 7. While for $\nu_{\text{HTA}} = 16$ MHz no ^{31}P signals were observed, for $\nu_{\text{HTA}}=5$ MHz clear negative signals were detected. For $\nu_{\text{HTA}} = 10$ MHz the negative signals were very weak. THYCOS measurements carried out on Mn- $^{15}\text{N}_5\text{ATP}$ with $\nu_{\text{HTA}} = 10$ MHz showed negative signals as well (see Fig. S6). These experiments indicate that for HTA pulse targeting EDNMR signals that exhibit unresolved hyperfine couplings that overlap with the central hole, as observed for the ^{14}N signals, the ^{31}P THYCOS spectrum has two contributions. One is a negative doublet arising from the HTA exciting allowed transition and the second is a positive doublet arising from non-selective excitation of the ^{14}N hyperfine components. Indeed, both spectra of Mn-AMPPNP and Mn-ATP contain positive and negative contributions, with Mn-AMPPNP having a stronger positive contribution. This is consistent with the significantly stronger ^{14}N signal of Mn-AMPPNP presumably due to the bridging ^{14}N between the phosphates. What remains unclear is the shift between the maxima of the negative and positive signals. Resolving this would require systematic measurements where the frequency of HTA pulse is varied by small steps through the complete ^{14}N signal. In the case of ^{14}N we have to consider also the nuclear quadrupole interaction, which adds more complications. The results of double- EDNMR reference experiment on Mn- P_i with excitation close to the central hole are shown in Fig. S9.

To substantiate the THYCOS and double-EDNMR results we also carried out simulations highlighting the ^{15}N - ^{31}P correlations. As mentioned in the Experimental section the simulations are carried out under the conditions that the HTA pulse saturates the forbidden transitions as opposed to inversion used in the schemes shown in Fig. S5. Inversion and saturation produce the same correlations.[38, 39] Fig. 8a shows three double-EDNMR spectra, where HTA1 pulse is set to the low and high frequency ^{15}N components as compared to a reference case with HTA1 pulse off-resonance to any signal in the EDNMR spectrum. Fig. 8b presents a zoom on the ^{31}P region after subtraction of the reference spectrum, where the ^{31}P doublet component that belongs the different electron-spin manifold than the ^{15}N hyperfine component excited by the HTA pulse is selected; the other component appears as a weak signal. This is the same as the behavior observed for the ^{55}Mn - ^{31}P correlation shown in Fig. 5b. More double-EDNMR simulations for various ^{15}N hyperfine couplings, showing the effect of the HTA pulse amplitude and of the overlap with the central hole are shown in Figs S10–S12. These calculations were done for ^{15}N and ^{31}P having positive hyperfine coupling (see Table 1, and recall that the gyromagnetic ratio of

^{15}N is negative) and they provide an correlation opposite to that observed for the experimental THYCOS results consistent with a negative ^{15}N coupling determined above.

Simulations of THYCOS highlighting the ^{15}N - ^{31}P correlation are shown in Fig. 8c. The bottom spectrum shows the calculated ENDOR spectrum, and the top traces show the calculated spectra for different frequencies of the HTA pulse. It shows a negative ^{31}P doublet when the HTA pulse frequency is shifted from the central hole by 5 MHz similar to the experimental observation with $\nu_{\text{HTA}}=5$ MHz (see Fig. 7). Setting $\nu_{\text{HTA}}=16.5$ MHz, the high frequency component of the ^{15}N doublet, selects high frequency ^{31}P doublet line, as in the double-ELDOR simulations and opposite to the experimental result. Setting $\nu_{\text{HTA}}=56.2$ MHz, on resonance with the low frequency doublet line of the ^{31}P doublet, generates a negative and positive signals as expected (see Fig. S5) [39].

DFT based simulations

DFT calculations were carried out to verify the stability of the Mn-ATP complex where both the N_7 and the phosphates are coordinated to the Mn^{2+} and to compare the DFT predicted ^{14}N hyperfine couplings with the experimental ones. The structure of ATP allows for a number of different coordination geometries for the metal ions. Given the spectroscopic data, we examined computational models involving simultaneous nitrogen and phosphate ligation. This introduced significant steric constraints. Manual modelling suggested that N_7 of the adenosine base was the only nitrogen atom that could reasonable satisfy a nitrogen and phosphate ligand sphere. Consequently, the starting models were manually constructed in which Mn^{2+} ion was ligated by the adenosine N_7 , two to three phosphate groups and sufficient water molecules to complete a quasi-octahedral geometry. Varying degrees of protonation of the triphosphate chain were also examined. The ensemble of starting structures were by no means exhaustive, but rather representative of the visually suitable geometries. Even so, many structures failed to achieve SCF convergence or geometry optimization criteria, likely due to highly unfavorable steric interactions. Some structures did achieve optimization, but had unrealistic geometries (for example, a highly distort 3 or 4-coordinate form involving a hydroxide ligand). None of the optimized structures were 6-coordinate. We found two optimized structures, referred to as structure 1 and structure 2, that were true energy minima. Both were square pyramidal (see Fig. 9a). In addition to N_7 , they also involved β - and γ -phosphate binding with the latter being protonated. One had two water molecules completing the ligand sphere while the other involved one water and the α -phosphate group as well. In both cases, a water ligand in the original quasi-octahedral starting-structure migrated away from the inner ligand sphere during the optimization process and formed hydrogen bonds with water and phosphate groups. The ^{31}P and ^{14}N hyperfine value obtained from the calculations are listed in Table 3. The ^{14}N isotropic hyperfine couplings predicted for the N_7 in both structures, 1.82 MHz for structure 1 and 2.50 MHz for structure 2, are on the order of the experimentally observed ^{14}N coupling, 2.3-3 MHz, with structure 2 showing a better agreement. Considering the negative gyromagnetic ratio of ^{15}N a positive ^{14}N coupling means a negative ^{15}N coupling consistent with the THYCOS results. Unsurprisingly, the biggest difference in the calculated hyperfine parameters of the two structures was the ^{31}P hyperfine interaction of the α -phosphate: in the directly ligated structure 1 the principal values of hyperfine tensor were

4.19, 4.68 and 7.75 MHz and in the other -0.22 , -0.27 and 0.90 MHz (Table 3). The coordination of N_7 leads to changes in the ^{31}P couplings, increasing those of α - ^{31}P and β - ^{31}P while increasing that of γ - ^{31}P (compare DFT values reported in Table 2 with those given in Table 3) [57]. The simulated ^{31}P ENDOR spectra of both structures compared with the experimental spectrum is depicted in Fig. 9b and the corresponding spectra of the individual ^{31}P nuclei are given in Fig. 9c. In general, the results of structure 1 with three coupled nuclei fits better, though both do not reproduce the shoulders at the inner region of the spectrum. In principle, it is possible for both configurations (two coupled phosphates and three) to coexist simultaneously in solution. Considering structure 1, where the Mn^{2+} is coordinated to all three phosphates and N_7 , the absence of any experimental evidence for the coordination of N_7 and the two phosphates in ADP remains puzzling because the strain is expected to be similar. Considering the large similarity of the ENDOR and EDNMR spectra of Mn-ATP and Mn-AMPPNP we conclude that they exhibit the same coordination sphere.

Conclusions

In this work we applied W-band THYCOS for determining the first coordination shell of Mn^{2+} in its complex with ATP. In particular, we showed through ^{15}N - ^{31}P correlations that a configuration where the Mn^{2+} is coordinated to the N_7 and phosphates is present in solution. We have carried out DFT calculations to verify the stability of such a configuration. The calculations generated two complexes where the N_7 is coordinated along with either all three phosphates or only P_γ and P_β . The calculated ^{14}N hyperfine couplings for both complexes were found to be on the same order of the experimental one. The experimental EDNMR spectra favor the coordination of all three phosphates. In contrast to Mn-ATP, for Mn-ADP we did not find evidence that Mn^{2+} coordinated to N_7 and phosphates. In addition, we expanded the understanding of the THYCOS experiment by comparing it to double-EDNMR for ^{55}Mn - ^{31}P correlation experiments and through simulations of THYCOS and double-EDNMR spectra for ^{15}N - ^{31}P correlations. Because of the low nuclear frequencies of ^{14}N and ^{15}N and their overlap with the central hole we explicitly explored THYCOS and double-EDNMR spectra obtained for HTA frequency region close to the allowed transition and found that this generates negative correlation signals arising from the off-resonance effects. Although such signals may overlap with the ^{14}N and ^{15}N correlation signal, they are weak and can be isolated. Carrying out these correlation experiments at frequencies higher than W-band should eliminate this problem.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- ENDOR and EDNMR were used to determine the coordination of Mn^{2+} to ATP and ADP.
- The correlation experiment THYCOS reveal the coupling of Mn^{2+} to both ^{15}N and ^{31}P .
- Mn^{2+} is coordinated to the adenosine N_7 and phosphates in Mn^{2+} -ATP.
- Simulations of THYCOS and double-EDNMR are presented.

Figure 1.

Possible coordination modes of Mn^{2+} to ATP: Coordination to both nitrogen and phosphate ($^{14}N-Mn^{2+}-^{31}P$) (a), only to phosphate ($Mn^{2+}-^{31}P$) (b) and only to nitrogen ($Mn^{2+}-^{14}N$) (c).

Figure 2:
Pulse sequences used in this work.

Figure 3. W-band ^{31}P Davies ENDOR the complexes of Mn^{2+} with ATP, AMPPNP and ADP (black traces). The spectra are overlaid with their respective simulations (red traces). The arrows mark the shoulders at ± 1 MHz.

Figure 4. W-band EDNMR spectra of Mn-ATP (black), Mn-¹⁵N₅ATP (green) Mn -AMPPNP (red) and Mn-ADP (blue). a) 150 MHz wide scan. b) Zoom on the ³¹P region. c) Zoom on the ¹⁴N/¹⁵N region. The intensity of the signals in the EDNMR spectra represents the ELDOR effect as described in the experimental section.

Figure 5.

a) EDNMR of the Mn-¹⁵N₅ATP sample. Arrows mark the position of the ⁵⁵Mn signals. b) THYCOS of the Mn-¹⁵N₅-ATP sample with the HTA pulse frequencies as noted on (a). c) Double-EDNMR spectra of the Mn-¹⁵N₅ATP. The ENDOR and EDNMR spectra are given as reference (black) in both (b) and (c), respectively. In (c) all spectra were multiplied by - to ease comparison of the EDNMR and ENDOR spectra.

Figure 6.

THYCOS spectra of the Mn-ATP, Mn-AMPPNP and Mn-ADP samples with the HTA pulse frequency set to $\nu_{\text{HTA}} = 10\text{MHz}$ ($\nu_L(^{14}\text{N})$). The spectrum of Mn- $^{15}\text{N}_5\text{ATP}$ was recorded with $\nu_{\text{HTA}} = 16.5\text{ MHz}$, matching the ^{15}N high frequency hyperfine component. Respective Davies ENDOR spectra are presented in red. THYCOS spectra recorded with different HTA pulse frequencies set to provide baseline can be found in SI Figure S7.

Figure 7.

THYCOS spectra of Mn-P_i for different frequencies of the HTA pulse as noted on the figure.

The Davies ENDOR of the ^{31}P region is given for comparison at the top. Comparison to the spectrum of Mn-ATP can be found in Figure S8.

Figure 8.

Simulation of hyperfine correlation spectra with the parameters listed in Table 1: a) Double-EDNMR simulated spectrum for the system of ^{15}N and ^{31}P with HTA1 set to the low ($\nu_{\text{HTA1}} = 12.1$ MHz, red) and high ($\nu_{\text{HTA1}} = 16.5$ MHz, black) frequency components of the ^{15}N hyperfine doublet. A powder average for the ^{15}N hyperfine coupling which is anisotropic was carried out. The blue trace corresponds to the HTA1 pulse set off resonance $\nu_{\text{HTA1}} = 300$ MHz. The insets zoom on the ^{15}N and ^{31}P regions. b) The ^{31}P region of the double-EDNMR simulations after the reference subtraction and multiplication by -1 . The traces were normalized before subtraction, same as was done for the experimental. c) ^{15}N and ^{31}P THYCOS simulated spectra for a single orientation. The microwave HTA pulse was set to one of the frequencies of one of the hyperfine lines of ^{15}N $\nu_{\text{HTA}} = 16.5$ MHz (red), of ^{31}P $\nu_{\text{HTA}} = 56.2$ MHz (blue), or to the side of the central hole at $\nu_{\text{HTA}} = 5$ MHz (magenta).

Figure 9.

(a) The DFT optimized structures 1 and structure 2. (b) Simulations of the ENDOR spectrum using the DFT predicted ^{31}P hyperfine couplings of Mn-ATP as compared to the experimental spectrum for both structures. (c) Simulated spectra (normalized) of the individual nuclei and their sum for structure 1 (c) and structure 2 (d).

Table 1.

Parameters used for the simulations of the double-EDNMR and THYCOS spectra.

Parameter description	Parameter designation	Value
Hahn echo (detection) pulse amplitude	ω_1 (det)	0.2 MHz
HTA pulse amplitude	ω_1 (HTA)	2.5 MHz
HTA pulse length	t_{HTA}	100 μs
^{31}P hyperfine principal values	$A_{31\text{P}}$	[3.5 3.5 5.5] MHz
^{14}N hyperfine principal values	$A_{14\text{N}}$	[0.5 0.5 1.1] MHz
^{14}N quadrupolar principal values	$P_{14\text{N}}$	[0.75 0.75 –1.5] MHz
^{15}N hyperfine principal values	$A_{15\text{N}}$	[4 4 9] MHz
Electron spin lattice relaxation time	T_{1e}	100 ms
Electron spin –spin relaxation time	T_{2e}	20 μs
Nuclear spin lattice relaxation time	T_{1i}	200 ms
HTA1 pulse frequency step	$d\nu$	0.1 MHz

Table 2:

Hyperfine parameters used for the simulations of the ENDOR spectra of the Mn^{2+} -nucleotide complexes and the values obtained from simulations based on DFT calculations, marked with *[57].

	Tentative assignment	A_{iso} (MHz)	T (MHz)	R (Å)
ADP	$\beta\text{-}^{31}\text{P}$	4.20	-0.82, -0.82, 1.64	3.4
ADP*	$\alpha\text{-}^{31}\text{P}$	4.1	-1.2, -0.3, 1.5	
	$\beta\text{-}^{31}\text{P}$	4.3	-1.4, -0.5, 1.9	
ATP	$\beta\text{-}^{31}\text{P}$	4.20	-0.71, -0.71, 1.42	3.6
	$\gamma\text{-}^{31}\text{P}$	3.45	-1.45, -1.45, -2.9	2.8
ATP*, bidentate	$\beta\text{-}^{31}\text{P}$	4.3	-1.3, -0.7, 2.0	
	$\gamma\text{-}^{31}\text{P}$	3.7	-1.5, -0.6, 2.2	
ATP*, tridentate	$\alpha\text{-}^{31}\text{P}$	4.2	-1.2, -0.7, 1.9	
	$\beta\text{-}^{31}\text{P}$	3.1	-1.2, -0.6, 1.8	
	$\gamma\text{-}^{31}\text{P}$	4.7	-1.3, -0.4, 1.7	
AMPPNP	$\beta\text{-}^{31}\text{P}$	4.15	-0.76, -0.76, 1.52	3.5
	$\alpha\text{-}^{31}\text{P}$	3.0	-1.15, -1.15, 2.3	3.0

Table 3:

DFT calculated hyperfine coupling parameters for Mn-ATP complex.

	T_{XX} , MHz	T_{YY} , MHz	T_{ZZ} , MHz	A_{iso} , MHz
Structure 1				
$\alpha\text{-}^{31}\text{P}$	-1.35	-0.86	2.21	5.54
$\beta\text{-}^{31}\text{P}$	-1.25	-0.81	2.05	4.29
$\gamma\text{-}^{31}\text{P}$	-0.35	-0.40	0.75	0.13
$^{14}\text{N}_7$	-0.45	-0.31	0.76	1.82
Structure 2				
$\alpha\text{-}^{31}\text{P}$	-0.22	-0.27	0.90	0.13
$\beta\text{-}^{31}\text{P}$	-1.14	-0.87	2.01	5.11
$\gamma\text{-}^{31}\text{P}$	-1.32	-0.57	1.9	4.98
$^{14}\text{N}_7$	-0.45	-0.34	0.78	2.50