# Metal ion-N7 coordination in a ribozyme branch domain by NMR

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Abstract: The N7 of purine nucleotides presents one of the most dominant metal ion binding sites in nucleic acids. However, the interactions between kinetically labile metal ions like Mg2+ and these nitrogen atoms are inherently difficult to observe in large RNAs. Rather than using the insensitive direct N-15 detection, here we have used (2)J-H-1,N-15]-HSQC (Heteronuclear Single Quantum Coherence) NMR experiments as a fast and efficient method to specifically observe and characterize such interactions within larger RNA constructs. Using the 27 nucleotides long branch domain of the yeast-mitochondrial group II intron ribozyme Sc.ai5 gamma as an example, we show that direct N7 coordination of a Mg2+ ion takes place in a tetraloop nucleotide. A second Mg2+ ion, located in the major groove at the catalytic branch site, coordinates mainly in an outer-sphere fashion to the highly conserved flanking GU wobble pairs but not to N7 of the sandwiched branch adenosine. 

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# Probing Metal Ion-N7 Coordination in a Ribozyme Branch Domain by NMR

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**The N7 of purine nucleotides presents one of the most dominant metal ion binding sites in nucleic acids. However, the detection of the interactions between kinetically labile metal ions like $Mg^{2+}$ and this site are inherently difficult to observe in large RNAs. The here described $^{2}J$-[$^{1}$H,$^{15}$N]-HSQC NMR experiments present a fast and efficient method to specifically observe and characterize such interactions within larger RNA constructs. Using the 27 nucleotides long branch domain of a group II intron ribozyme as an example, we show that direct N7 coordination takes place in a tetraloop nucleotide, whereas a $Mg^{2+}$ ions binds majorly to the flanking GU wobble pairs but not the sandwiched branch adenosine.**

RNAs are inextricably linked to metal ions. Hence, all ribozymes can be considered to be obligate metallo-ribozymes depending strongly on the presence of monovalent as well as divalent metal ions.[1] In order to understand the role of $M^{n+}$ ions in folding and catalysis of large RNAs like group II intron ribozymes,[2,3] the exact coordination sphere of crucial ions for these processes needs to be known. However, it is highly challenging to determine the exact coordination sphere of kinetically labile metal ions in larger RNA structures in solution state. From NMR studies on the nucleobases it is well known that metal ion coordination to, e.g., N7 leads to a downfield shift of the adjacent H8.[4-6] In larger RNA structures, the situation is more complex, as aside from metal ion binding, also the binding mode, e.g., inner *versus* outer sphere, as well as small structural changes affect the proton chemical shifts. Metal ion coordination to bridging phosphate moieties has been probed by thiophosphate modification,[7-12] but for the nucleobase residues the situation is more complicated. The N7 of purine bases is thereby of special interest as this atom constitutes a prominent coordination site for metal ions in nucleic acids.[5,6,13-15] The ring nitrogens of the purine bases in the hammerhead ribozyme have been probed for $M^{n+}$ binding by observing the change of $^{15}$N chemical shift using direct $^{15}$N detection.[16] Recently, the coordination of $Hg^{2+}$ to N3 of thymine has been proven by the same method by incorporating specifically labelled nucleotides into a DNA duplex.[17] However, due to its low gyromagnetic moment, direct $^{15}$N detection is very time consuming and the assignment of the peaks in a $^{15}$N spectrum of a large RNA rather complicated. Here, we present a fast end efficient method to simultaneously observe the change in chemical shifts changes of the purine H8 and N7 resonances upon $Mg^{2+}$ coordination making it possible to distinguish direct $Mg^{2+}$ coordination from other effects.

For our studies we chose a 27 nucleotide long domain 6 construct of a group II intron ribozyme derived from the mitochondrial intron Sc.ai5γ from *Sacharomyces cerevisiae*. Domain 6 contains the nucleophile for the first step of splicing, i.e. the 2'-OH of a conserved adenosine, of these selfsplicing ribozymes, which exhibit a pathway strongly resembling the one of the

spliceosomal machinery.[18-20] Like all large ribozymes investigated to date, group II introns are obligate metalloribozymes that require $Mg^{2+}$ for folding and catalysis.[21-27] A recent crystal structure of a group IIC intron from *Oceanobacillus iheyensis* depicts the global architecture although domain 6 is disordered in the crystal.[28] Several $Mg^{2+}$ ions were identified, two of them 4 Å apart in the catalytic core neighbouring domain 5 (D5). These findings suggest a two-metal ion mechanism, one of these ions activating the nucleophilic 2'-OH. Recent solution NMR studies have revealed four metal ions bound site specifically to D6-27, one of them being located in the major groove at the branch adenosine and the two flanking GU wobble pairs (Fig. 1).[29,30] However for this site, as well as for the whole group II intron architecture, the exact coordination spheres of $Mg^{2+}$ ions remain elusive.

Line broadening studies with $Mg^{2+}$ and $Mn^{2+}$ have indicated metal ion binding within the branch region, i.e. at A20 and the flanking GU wobble pairs, the 5'-terminus, the tetraloop and the two helical regions.[30] In order to identify direct N7 coordination of $Mg^{2+}$ at these sites, we performed a series of $^{2}J$-[$^{1}$H,$^{15}$N]-HSQC experiments[31,32] that were optimized for the 11.4 Hz coupling between H8 and N7 of purine bases. Thereby we used two D6-27 constructs in which either all adenosine or all guanosine nucleotides were $^{13}$C,$^{15}$N isotope labelled. All H8-N7 crosspeaks in the two types of spectra are well separated allowing unambiguous assignment. The resonances of the first three nucleotides at the 5'-end of D6-27, i.e. G1, G2, and A3, are split into two sets of signals due to the presence of either a terminal triphosphate (TP) or a diphosphate (DP) chain as has been discussed previously.[30] Increasing amounts of $Mg^{2+}$ were then added in six steps from 0 – 2.5 mM to follow the change in chemical shift as well as the line width of the N7 and H8 resonances allowing to investigate $Mg^{2+}$-N7 coordination in detail (Fig. 2).

Remarkably, all resonances change their chemical shift either in the $^{15}$N or $^{1}$H dimension, or both, indicating manifold interactions of the $Mg^{2+}$ ions with D6-27 and possibly $Mg^{2+}$ induced structural changes. Most resonances are not significantly broadened upon addition of $Mg^{2+}$, the N7 of A14 being the notable exception. Line broadening upon binding of the diamagnetic $Mg^{2+}$ is a well-known effect,[30,33] which has for example been observed in $^{31}$P-NMR studies of ATP derivatives.[11] This effect can be attributed to the fast on- and off-rates of first-shell ligand-exchange of $Mg^{2+}$, which is in the order of the NMR time scale. The substantial line broadening effect observed at A14N7, which is located in the tetraloop, could thus be a direct consequence of inner-sphere binding to this site. The large upfield shift of G12N7 (–1.51 ppm) corroborates $Mg^{2+}$ binding in this region. Metal ion binding to tetraloop structures is well known,[34] and is also well in line with the previously determined affinity constant log $K = 2.14 \pm 0.03$ of $Mg^{2+}$ to this site.[30] In contrast, the absence of line broadening for all other H8-N7 resonances within D6-27 suggests that these purine N7-sites are predominantly outer-sphere coordinated by $Mg^{2+}$.

Aside from $Mg^{2+}$ binding to the tetraloop, further specific binding sites have been identified at the terminal phosphate groups, the two helical as well as the branch regions.[30] All these regions contain numerous adenosine and guanosine residues allowing us here to explore N7 coordination. The split resonances of the first three nucleotides at the 5'-end all show at least small changes in chemical shift of both nuclei. The $G2_{DP}$ resonance is most strongly upfield shifted in $^{1}H$, which is in contrast to the other resonances of these three nucleotides showing only little changes in chemical shifts in the $^{1}H$ dimension. On the other hand, the $N7G2_{DP}$ resonance is the one least affected by $Mg^{2+}$, suggesting no direct $Mg^{2+}$ binding at this atom. The two helical regions of D6-27 are predominantly composed of GC base pairs. All H8-N7 resonances of the two helical stems display only a minimal change in chemical shift in the $^{1}H$ dimension, but a much more pronounced upfield shift of the $^{15}N$ resonance. Taken together, the large changes in chemical shifts of the N7 resonances in these three regions confirm previous findings of $Mg^{2+}$ coordination to the purines.

$Mg^{2+}$ binding to the branch region is certainly of utmost interest for the elucidation of the catalytic mechanism of group II intron ribozymes. Remarkably, the N7 resonance of the branch adenosine A20 is the only one within D6-27 that is invariant to the addition of $Mg^{2+}$, whereas a large downfield shift of the neighbouring A20H8 is observed (Fig. 2b). On the other hand, the resonances of the two N7 sites of the neighbouring guanosines G7 and G8, both involved in GU wobble pairs, are very strongly affected (Fig. 2c). In fact, N7 of G8 displays the largest $\Delta\delta = -1.87$ ppm of the whole D6-27 construct. These findings suggest that the $Mg^{2+}$ ion at the branch site coordinates to the two flanking GU wobble pairs, but not to the N7 position of the branch adenosine itself. This is corroborated by previous $Mn^{2+}$ line broadening experiments, where only an effect at the H8 protons of G7 and G8 has been observed.[30] The large $\Delta\delta = 0.18$ ppm in the $^{1}H$ dimension of A20H8 can thus be attributed to a change in local structure of the branch site: The downfield shift indicates a reduced stacking interaction with the neighbouring nucleobases. This effect can be explained by a tightening of the branch site due to the coordination of $Mg^{2+}$ to the two GU wobble pairs, and a subsequent increased exposure of the branch adenosine to the solution.

Changes in chemical shifts upon metal ion binding within a larger nucleic acid are very difficult to interpret as they can be the results of several factors: Upon coordination of a metal ion to N7, H8 is shifted downfield due to the electron pulling effect of the coordinating metal ion. However, an accompanying structural change, e.g. increased stacking interaction leads to a simultaneous upfield shift. Here, the described extension into the $^{15}N$ dimension adds important information: with the notable exceptions of A15 and A20, all N7 resonances are upfield shifted upon addition of $Mg^{2+}$. An upfield shift of the coordinating nitrogen has been observed earlier in

the case of $Hg^{2+}$ mediated thymine-thymine base pairing within a DNA helix,[17] where the metal ion coordinates directly to the N3 position. However, in the latter case, the $Hg^{2+}$ ion replaces two $H^+$ ions, explaining the upfield shift. In contrast, in the case of D6-27, no protons are replaced upon $Mg^{2+}$ binding. Instead, the upfield shift could very well mean that $Mg^{2+}$ coordination to the N7 positions takes place in an outer sphere fashion, i.e. mediated via a coordinated water molecule. Such a coordination mode is very common for this metal ion. Based on the NMR structure, the four carbonyl oxygens and two N7 positions of the GU wobble pairs flanking the branch adenosine, all lying in the major groove, are between 5 and 9 Å apart. Taking the shape of the binding pocket into account, such distances could only be bridged by mainly outer sphere binding. Hence, we suggest that a $[Mg(H_2O)_n]^{2+}$ complex binds specifically to the phosphate oxygens and especially the carbonyl oxygens and purine N7 positions of the flanking GU wobble pairs of the branch adenosine.

In order to understand the role of metal ions in ribozyme catalysis as well as their specific accelerating or inhibiting effect,[3,35-37] their exact coordination sphere needs to be known. The $Mg^{2+}$ ion sitting in the major groove of the branch adenosine is close enough to exhibit a crucial electrostatic effect on the branching reaction.[1] Our results here now provide the first information on the liganding sites of this ion on an atomic level. The $^2J$-[$^1$H,$^{15}$N]-HSQC NMR experiments used in the current study are very well applicable to any larger nucleic acid structure yielding important information in short experimental time.

## EXPERIMENTAL SECTION

### Materials and Instrumentation

DNA oligonucleotides were purchased from Microsynth, Balgach (Switzerland) or from Operon, Cologne (Germany). The nucleotide triphosphates came from Amersham Biosciences Europe GmbH, Otelfingen (Switzerland), except for UTP, which was obtained from Sigma-Aldrich-Fluka, Buchs (Switzerland). $^{13}$C,$^{15}$N-labeled NTPs were from Silantes GmbH, München (Germany). T7 polymerase used for *in vitro* transcription was homemade.[38,39] For desalting Vivaspin 2 from Vivascience (Sartorius) with a cutoff of 3000 MWCO were used. $MgCl_2$ for the metal ion titration was obtained as 1 M ultrapure solution in $H_2O$ from Fluka. The exact concentrations of the $MgCl_2$ stock solutions in 99.999% $D_2O$ each (Sigma-Aldrich) was determined by potentiometric pH titration employing EDTA. The Sep-Pak Vac 6cc (500 mg) C18 cartridges for removal of inorganic ions from the RNA after titration were from Waters Corporation (Milford, Massachusetts, USA). All chemicals used were at least puriss p.a. and purchased from either Fluka-Sigma-Aldrich or Brunschwig Chemie, Amsterdam (The

Netherlands). UV measurements to determine the RNA concentrations were carried out on a Varian Cary 500 Scan UV-VIS-NIR spectrophotometer using a 10 mM QS cuvette (Hellma, Müllheim, Germany). NMR spectra were recorded on a Bruker AV600 MHz spectrometer equipped with a TCI z-gradient CryoProbe or an AV700 MHz spectrometer equipped with a TXI z-gradient CryoProbe at the NMR facility of the Chemistry Department at the University of Zurich.

**NMR sample preparation**

D6-27 (5'-GGAGCGGGGGUGUAAACCUAUCGCUCC) was synthesized by *in vitro* transcription with T7 polymerase from a double stranded DNA template.[39] The reaction mixture contained 5 mM of each NTP, 0.9 μM of the double stranded DNA template, 0.1 % Triton X-100, 40 mM Tris-HCl (pH 7.5), 40 mM DTT, 2 mM spermidine and 30 mM $MgCl_2$. The amount of T7 polymerase was optimized individually for each new polymerase batch. Natural isotope abundance as well as fully or partly $^{13}C$,$^{15}N$-enriched samples of both constructs were transcribed and used for NMR measurements. All RNA was purified by denaturing 18% PAGE, UV-shadowed, excised from the gel and recovered by electroelution. Desalting was done by ultrafiltration at 3000 g. After lyophilisation, the sample was dissolved in 220 μL $D_2O$ (50 mM KCl, 10 μM EDTA, pD 6.7). To measure the pD value, 0.4 log units were added to the pH meter reading.[40,41] The RNA concentration was determined by UV-VIS spectrophotometry, using an extinction coefficient at 260 nm ($\varepsilon_{260}$) of 296.3 $mM^{-1}cm^{-1}$ for D6-27. The concentrations of the RNA samples varied between 0.5 and 1.57 mM. All samples were lyophilized and resuspended in either 90% $H_2O$/10% $D_2O$ or 99.999% $D_2O$ prior to acquisition of NMR spectra.

**NMR spectroscopy**

$^2J$-[$^1$H,$^{15}$N]-HSQC experiments[31,32] with a selective 180° $^{15}$N IBurp2 pulse of 3 ms and simultaneous $^{15}$N-$^{13}$C decoupling were recorded to observe the $^2J$-coupling between H8 and N7 of purine bases. The titration series monitoring the N7-H8 crosspeaks of all adenines in D6-27 were performed on a 0.55 mM RNA sample where all adenines were $^{13}C$,$^{15}N$ labelled. The sample was dissolved in 100 % $D_2O$ containing 50 mM KCl at a pD of 6.7. The experiments were performed at 298 K in presence of 0, 0.5, 1, 1.5, 2 and 2.5 mM $MgCl_2$. The guanosine N7 resonances were monitored with a D6-27 sample, where all guanosines were $^{13}C$,$^{15}N$ labelled. The RNA was again dissolved in 100% $D_2O$ with 50 mM KCl at pD = 6.8. The titration was performed with 0, 0.5, 0.75, 1, 1.5, 2, and 2.5 mM $MgCl_2$. At 2.5 mM $MgCl_2$ the average line width had broadened severely and thus these data points were not included in the quantitative analysis of the chemical shift changes.

All NMR spectra were processed with TOPSPIN 2.0 (Bruker BioSpin) and analyzed with Sparky (http://www.cgl.ucsf.edu/home/sparky/).

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**FIGURE CAPTIONS**

**Fig. 1** A schematic view of the common secondary structure of group II intron ribozymes with the six domains (D1, D2, D3, D4, D5 and D6) projecting from a central wheel. D1 is further partitioned in four subdomains a, b, c, d. The intron- and exon binding sites (IBS and EBS respectively) are indicated in magenta. The branch-domain D6 is highlighted in red and enlarged in 3D as the solution structure of D6-27.[29] The branch-adenosine that is stacked within the helix is highlighted in red, the flanking GU-wobble pairs in gold. All previously determined metal ion binding sites are indicated.[30] Above the solution structure, the sequence of D6-27 is given (colour code as mentioned).

**Fig. 2** Section of $^{2}J$-[$^{1}$H,$^{15}$N]-HSQC spectra with a selective 180° $^{15}$N IBurp2 pulse to monitor the $^{2}J$ coupling between H8 and N7 of purine nucleotides in D6-27. Experiments were recorded with increasing $Mg^{2+}$ concentration in 0.5 mM steps from 0 – 2.5 mM. (a) Observed $^{2}J$ coupling between H8 and N7, as shown in the case of a adenine nucleobase. (b) The N7 chemical shift of A20 is unperturbed, whereas its neighbouring H8 is strongly affected (white arrow) indicating a positional change of this nucleobase, but no direct $Mg^{2+}$ coordination. All other N7 resonances positions are affected. (c) The guanine N7 chemical shifts of the two branch GU wobble pairs (white arrows) are strongly affected by $Mg^{2+}$ indicating a $Mg^{2+}$ interaction with these atoms.

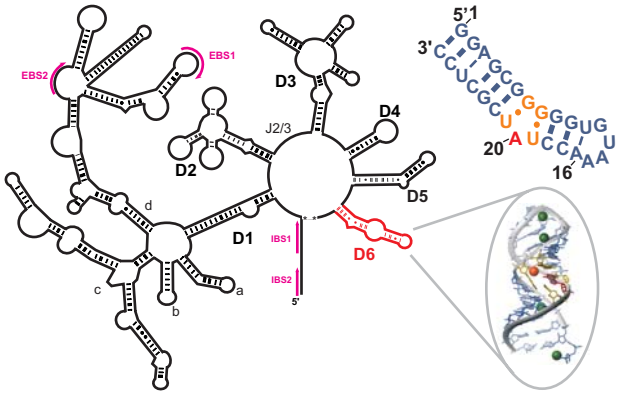
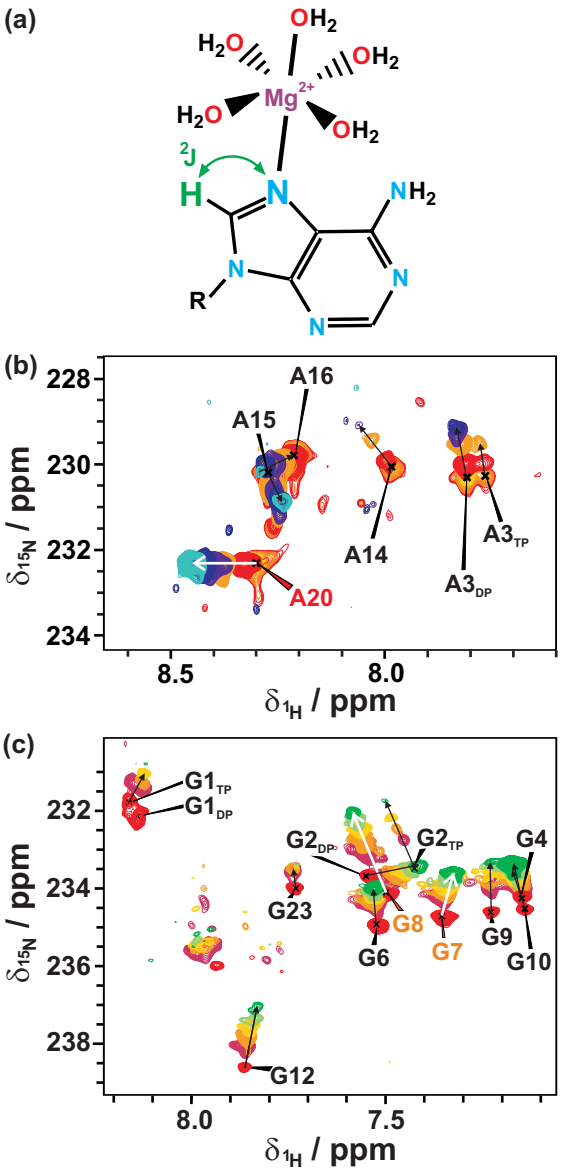



Figure 1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OH2
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N
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N
N
R
N
(b)
228
230
232
234
δ15N / ppm
A16
A15
A14
A20
A3TP
A3DP
8.5
8.0
δ1H / ppm
(c)
232
234
236
238
δ15N / ppm
G1TP
G1DP
G2DP
G2TP
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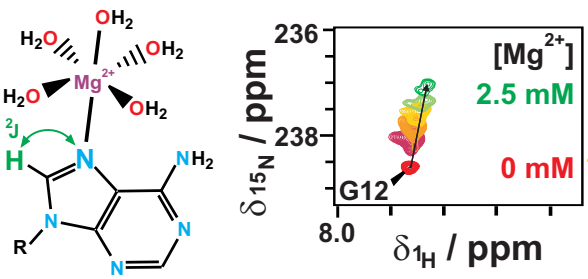
δ1H / ppm

**Figure 2**




**Graphics for**

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