Metal ion binding to an RNA internal loop

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Abstract

Studying the interaction of metal ions with RNA is challenging because of the fast dynamics of the system and the intricate interplay between structural and functional roles of metal ions. NMR spectroscopy is an exceptional tool to investigate such interactions in solution and allows for a detailed description of both metal ion binding sites and binding modes in complex and dynamic RNA structures. We recently applied heteronuclear NMR to study the metal ion binding properties of a three-way junction RNA (D1 $\kappa\zeta$) which plays an important role in group II intron splicing, and observed metal ion binding in both κ and ζ regions of the construct. Here we concentrate in more detail on the ζ region (D1 ζ) using NMR to investigate the interaction with Mg(II), Cd(II) and cobalt(III)hexamine. Our data confirm Cd(II) induced macrochelate formation at the 5'-end triphosphate, suggest an overall similar behaviour for the two divalent metal ions, but with much clearer changes in chemical shifts upon Cd(II) addition, and reveal only little changes upon cobalt(III)hexamine addition, allowing to discriminate between inner- and outer-sphere binding. Moreover, we observed distinct differences when we titrated the sample with Cd(II) in the presence of either KCl or KClO₄ as background monovalent salt.

Keywords: RNA; metal ion binding; NMR

Introduction

Metal ions are intrinsically associated with RNA due to its polyanionic nature [1, 2]. They screen the overall negative charge of the polyphosphate backbone mainly by diffusive binding, but they can also be located in specific binding pockets, and eventually be involved in catalysis [3]. We have recently reported about the role of Mg(II) in the stabilization of the D1 $\kappa\zeta$ region of the mitochondrial group II intron ribozyme *Sc.ai5 γ* from baker's yeast [4]. Group II intron ribozymes are large catalytically active non-coding RNAs able to perform self-splicing without the aid of a protein [5, 6]. Their mechanism of splicing resembles the one of the spliceosome, the complex molecular machine responsible for mRNA maturation in eukaryotes. Studying structure, activity and metal ion binding of these RNAs may thus also provide a better understanding of the

spliceosome activity [7, 8]. Although group II introns comprise several hundreds of nucleotides arranged in six domains (Figure 1a) the catalytic core is represented by domain 5 [9] interacting with a small region within the large domain 1, encompassing the κ and ζ elements [10, 11]. We used NMR spectroscopy to solve the structure of the isolated D1 $\kappa\zeta$ molecule [4]. The construct proved to be dynamic in solution and Mg(II) was shown crucial to stabilize the structure allowing coaxial stacking between the two helices d' and d'' (Figure 1b). Given the importance of metal ions in stabilizing this RNA construct we successively investigated metal ion binding sites and modes to this construct employing heteronuclear NMR spectroscopy [12].

Figure 1: a) Secondary structure of the group II intron *Sc.ai5γ* from *S. cerevisiae* with the $\kappa\zeta$ region highlighted. The six domains (D1-D6) originate from a central wheel and domain 4 may contain an open reading frame (ORF). b) D1 $\kappa\zeta$ secondary structure. c) D1 ζ secondary structure. The ζ region is highlighted in black and the κ region in grey. A UUCG tetraloop was added to stabilize the constructs D1 $\kappa\zeta$ [4] and D1 ζ , while guanosines were added at the 5'-end to increase transcription yield [13]. These additional nucleotides are indicated with asterisks.

We used ^1H , ^{13}C , ^{15}N and ^{31}P NMR to follow chemical shift changes of the RNA residues in the presence of Cd(II) and cobalt(III)hexammine and we compared the results with the behaviour observed upon addition of Mg(II). The use of Cd(II) and cobalt(III)hexammine as inner-sphere and outer-sphere binding mimics of Mg(II), respectively, is well acknowledged (Figure 2) [13-15].

Cd(II) normally binds to RNA with slightly higher affinity than Mg(II), likely by inner-sphere coordination, and with better observable and sharper NMR peaks, due to its fast solvent exchange kinetics. On the other hand, cobalt(III)hexammine, with its kinetically inert ligands, has been widely used to investigate outer-sphere binding sites [1].

Figure 2: Inner-sphere coordination (left) and outer-sphere coordination (right) of metal ions (**M**) to a guanine-N7 are depicted. The 2J coupling observed between, e.g., N7 and H8, with [^1H , ^{15}N]-HSQC experiments is indicated.

Our results revealed the presence of a Cd(II)-assisted macrochelate formation at the 5'-end triphosphate and several binding sites located mostly in non-canonical RNA regions, including the GAAA tetraloop and the κ element. Also, a general electrostatic interaction at the ζ tetraloop receptor was observed. However, with exclusion of the strongest binding at the 5'-end, the other binding sites seem to compete equally for metal ion interaction making a detailed investigation difficult [12]. For this reason, we decided to apply heteronuclear NMR to study metal ion binding of a reduced D1 ζ construct (Figure 1c), which contains only the internal loop and no three-way junction. This internal loop acts as a tetraloop receptor for the GAAA tetraloop in the context of the whole group II intron [10, 11]. The GNRA tetraloop-receptor interaction indeed represents a crucial RNA folding motif [16]. A number of studies have been reported on this highly conserved 11nt asymmetric internal loop, which differs from our construct only for the inversion of a C-G base for a G-C base, being this a common natural mutation [16]. The rest of the sequence corresponds to the general conserved receptor, and comprises the asymmetric internal loop, which presents three adenines in a zipper-like stacking capped by a U-U mismatch and a G-U wobble base pair [4, 17].

Conformational rearrangement of the receptor was observed upon tetraloop binding [17-19]. The GAAA/tetraloop receptor system serves as a model to study metal ion-RNA interaction [16] and studies on metal ion binding to the GAAA tetraloop – 11nt receptor have been published [16, 20, 21]. No effect on imino proton signals of the isolated tetraloop receptor was observed upon Mg(II) titration [17], suggesting that Mg(II) does not induce any structural change. Similarly, no difference was observed in the structure of the tetraloop receptor docked to a GAAA tetraloop in the presence of a variety of cations [20]. In the docked form, the AA platform in the asymmetric internal loop was suggested to be a monovalent metal ion binding site based on the crystal structure of the *Tetrahymena* P4–P6 domain [21]. The same crystal structure suggested also Mg(II) binding to the oxygen phosphate of G8 (D1 ζ numbering). These two binding sites in the asymmetric loop were confirmed by NMR studies [20]. Interestingly, metal ions seem to localize in regions of highly negative density, rather than in defined binding pockets. Similar conclusions on general electrostatic interaction at the asymmetric internal loop were drawn by our recent study, where metal ion binding to the undocked tetraloop receptor in D1 $\kappa\zeta$ was investigated by NMR [12]. Given the ability of heteronuclear NMR to give a precise description of metal ion binding sites and modes and the interest in such structural element, we here performed a detailed NMR study of metal ion binding of the isolated tetraloop receptor (D1 ζ). By a combination of [^1H , ^{15}N]-HSQC (heteronuclear single quantum coherence), ^{31}P and ^{113}Cd spectra, the behaviour of D1 ζ in the presence of Mg(II), Cd(II) and cobalt(III)hexammine was investigated in detail. Line broadening experiments in the presence of Mn(II) were also recorded.

Material and methods

DNA oligonucleotides were acquired from Microsynth (Balgach, Switzerland). The nucleoside 5'-triphosphates (NTPs) were purchased from Carl Roth GmbH (Karlsruhe, Germany) except for ATP, which was from GE Healthcare (Glattbrugg, Switzerland). ^{15}N -labelled NTPs were acquired from Silantes GmbH (München, Germany). T7 polymerase used for in vitro transcription was home-made [22]. For electroelution the Elutrap System with BT1 and BT2 membranes (Whatman®) was

used. Ultrafiltration devices Vivaspin 2-mL with 3 kDa molecular weight cut off were purchased from Sartorius Stedim biotech. (Aubagne, France). $^{113}\text{Cd}(\text{NO}_3)_2$ was prepared from a reaction of $^{113}\text{CdCl}_2$ (Cambridge Isotope Laboratories, Burgdorf, Switzerland) and 1.9 equiv. of AgNO_3 (Acros-Organics, Geel, Belgium). The exact concentration of the ^{113}Cd stock solution in 100 % D_2O (Armar Chemicals, Döttingen, Switzerland) was measured by atomic absorption spectroscopy (AAS). $[\text{Co}(\text{NH}_3)_6]\text{Cl}_3$ was synthesized in-house according to the published protocol [23] and the concentration was measured by UV-Vis ($\epsilon_{476 \text{ nm}} = 56.5 \text{ M}^{-1}\text{cm}^{-1}$ and $\epsilon_{340 \text{ nm}} = 46.1 \text{ M}^{-1}\text{cm}^{-1}$). The concentration of MgCl_2 and MnCl_2 stock solutions in D_2O was determined by potentiometric pH titration with EDTA [24].

RNA sample preparation

The 27-nucleotide long RNA samples were synthesised by in vitro transcription with T7 polymerase from double-stranded DNA [13]. Transcription reactions were generally allowed to proceed for 5 hours. The DNA template used contained 2'-methoxy groups on the two 5'-terminal guanosines, to avoid 3'-end inhomogeneity [25]. All transcribed RNA samples were purified by PAGE (15%) and recovered by electroelution. After desalting by ultrafiltration in Vivaspin® devices and lyophilization, the RNA samples were dissolved in 240–250 μL of 60 mM KCl or KClO_4 , 10 μM EDTA and D_2O , and transferred into Shigemi® tubes. The final concentration of the samples prepared for NMR measurements was determined by UV-Vis ($\epsilon_{260 \text{ nm}} = 266 \text{ mM}^{-1}\text{cm}^{-1}$). The concentration of the samples varied between 0.4–1.2 mM and the pD between 6.7 and 7.1.

NMR spectroscopy

NMR spectra were recorded on a Bruker Avance 700 MHz spectrometer with a 5 mm TXI CryoProbe™ and a Bruker Avance 500 MHz equipped with a 5 mm BBO CryoProbe™ at 300 K. ^1H NMR, ^{31}P NMR, ^{113}Cd NMR, 2J - $[^1\text{H}, ^{15}\text{N}]$ -HSQC, $[^1\text{H}, ^{31}\text{P}]$ -HSQC-NOESY and $[^1\text{H}, ^1\text{H}]$ -NOESY spectra were recorded and processed using Topspin 3.0 (Bruker BioSpin) and analysed with Sparky (<http://www.cgl.ucsf.edu/home/sparky/>). ^1H NMR spectra were measured with water suppression and referenced to external 2,2-dimethyl-2-silapentane-sulfonic acid (DSS, 0.2%, pH 7.5). ^{15}N

chemical shifts were indirectly referred to ^1H of DSS [26], ^{113}Cd NMR spectra were referenced to external 0.1 M $\text{Cd}(\text{ClO}_4)_2$ [27] and ^{31}P NMR spectra were referenced to external 85 % H_3PO_4 .

Cd(II), cobalt(III)hexamine, Mg(II) and Mn(II) titrations

^{15}N labelled D1 ζ samples in the presence of 60 mM KCl or KClO_4 were titrated with increasing amounts of $^{113}\text{Cd}(\text{NO}_3)_2$ in 0.1 to 0.5 mM steps to a final Cd(II) concentration of 3 mM. ^1H NMR, 2J - $[^1\text{H}, ^{15}\text{N}]$ -HSQC and ^{113}Cd NMR spectra were recorded at each step of metal ion addition. Natural abundance samples in the presence of 60 mM KCl were used to assign the backbone phosphates by $[^1\text{H}, ^{31}\text{P}]$ -HSQC-NOESY experiment and were then titrated with increasing amounts of $^{113}\text{Cd}(\text{NO}_3)_2$ to 0.9, 1.8 and 6 equiv. of Cd(II). Similarly, a ^{15}N labelled D1 ζ sample in the presence of 60 mM KCl was titrated with cobalt(III)hexamine in 0.2 to 0.5 mM steps to a final concentration of 3 mM and a ^{15}N labelled sample in the presence of 60 mM KCl was titrated with MgCl_2 in 0.35 to 2 mM steps to a concentration of 12 mM. ^1H and 2J - $[^1\text{H}, ^{15}\text{N}]$ -HSQC experiments were recorded after each metal ion addition. Natural abundance samples in the presence of 60 mM KCl or KClO_4 were titrated with $^{113}\text{Cd}(\text{NO}_3)_2$, cobalt(III)hexamine and MgCl_2 in 0.1 to 1 mM steps to a final metal ion concentration of 3 mM. ^{31}P NMR spectra were measured at each step. A natural abundance sample in the presence of 60 mM KCl was titrated with MnCl_2 in three steps, to 30, 60, and 120 μM concentration. ^1H NMR and $[^1\text{H}, ^1\text{H}]$ -NOESY spectra were recorded at each step.

Results

2J - $[^1\text{H}, ^{15}\text{N}]$ -HSQC spectra to study metal ion binding

2J - $[^1\text{H}, ^{15}\text{N}]$ -HSQC experiments were used as key approach to study the interaction [4, 12, 13, 28, 29]. Such experiments allow to contemporary follow chemical shift changes in N7/N9/H8 and N3/N1/H2 resonances. These experiments are of particular interest since purine N7 often represents a direct binding site for metal ions [3, 29] and inner-sphere interaction often results in strong upfield shift of ^{15}N resonance [30, 31]. ^{15}N labelled RNA samples were titrated with increasing amounts of the three metal ions. Representative titrations are shown in Figure 3.

Figure 3: Overlay of 2J - $[^1\text{H}, ^{15}\text{N}]$ -HSQC spectra of a) 0.64 mM ^{15}N labelled D1 ζ titrated with increasing amounts of Cd(II) (60 mM KCl, pD 6.7, 300 K, 500 MHz, b) 0.9 mM ^{15}N labelled D1 ζ titrated with increasing amounts of Mg(II) (60 mM KCl, pD 6.8, 300 K, 700 MHz), c) 0.55 mM ^{15}N labelled D1 ζ titrated with increasing amounts of cobalt(III)hexamine (60 mM KCl, pD 7.0, 300 K,

700 MHz), d) 0.55 mM ^{15}N labelled D1 ζ titrated with increasing amounts of Cd(II) (60 mM KClO_4 , pD 6.9, 300 K, 700 MHz). Squares indicate line broadening and coloured arrows indicate a significant induced $\Delta\delta$ upon addition of Cd(II) (blue) and Mg(II) (green) in the presence of KCl. The red arrow in d) shows the different behavior of A22N1 resonance observed in the presence of KClO_4 compared to KCl in a Cd(II) titration.

The peaks are well resolved, allowing safe **assignment** of all the resonances (Tables S1-S4) in accordance with previously published values for D1 $\kappa\zeta$ [12], **thus confirming similar arrangement of the internal loop**. Moreover, thanks to good spectral dispersion, the chemical shift changes upon metal ion addition could be easily followed. In the case of Cd(II) addition (Table S1), strong N7 chemical shift variation is observed at G1 with a $\Delta\delta$ of around 22 ppm suggesting macrochelate formation at the 5'-end as previously observed for D1 $\kappa\zeta$ [12]. Strong changes in N7 chemical shift are observed for G2 ($\Delta\delta \sim 15$ ppm), G16 and G18 ($\Delta\delta \sim 13$ and 7 ppm, respectively), confirming the ability of Cd(II) to bind guanine residues. The binding is also reflected in the shift of N9 resonance (Figure 3a). The N7 chemical shifts of A3 and A17 also undergo significant changes ($\Delta\delta \sim 5$ in the two cases) while few changes are observed for N1, N3 and H2 resonances of residues in helical regions. The resonances of the three adenines of the internal loop also show some changes with A22 being the most affected residue (N3 chemical shift moves downfield by ~ 2 ppm and N1 chemical shift moves upfield by ~ 4 ppm). Much smaller chemical shift variations are observed in the presence of increasing amounts of Mg(II) (Table S2). The $^2J\text{-}[^1\text{H}, ^{15}\text{N}]$ -HSQC spectra (Figure 3b) show indeed significant chemical shift changes only at the 5'-end (N7 $\Delta\delta \sim 4$ ppm), which eventually broadens under detection at 4.6 equiv. of Mg(II), and at A22-N1 in the internal loop ($\Delta\delta \sim 2$ ppm), which shows a similar trend of $\Delta\delta$ as previously observed with Cd(II). Little or no chemical shift changes are observed with cobalt(III)hexammine (Table S3, Figure 3c). It is worth noting that A6 and A21 in the internal loop are somehow similarly affected by the three metal ions, which cause, for example, a $\Delta\delta$ of ~ 0.5 -1.5 in the N3 residue. (Figure 3).

Cd(II) titration performed in KClO_4 : $^2J\text{-}[^1\text{H}, ^{15}\text{N}]$ -HSQC and ^{113}Cd spectra

The experiments commented in the previous paragraph were recorded in KCl. However, it is known that chloride ions interacts with Cd(II) to form a stable CdCl^+ complex in solution, which may bind

to RNA [2]. For example, an early study on CdCl_2 and $\text{Cd}(\text{ClO}_4)_2$ interaction with ATP shows that $\text{Cd}(\text{II})$ and CdCl^+ species have considerably different behaviour towards ATP, and this influences also their ^{113}Cd chemical shift [32]. Moreover, the formation of CdCl^+ would lead to an overall decrease of the $\text{Cd}(\text{II})$ available for RNA binding in solution [2]. For this reason, the $\text{Cd}(\text{II})$ titration was repeated in the presence of 60 mM KClO_4 . The $^2J\text{-}[^1\text{H}, ^{15}\text{N}]\text{-HSQC}$ spectra recorded in the presence of increasing amounts of $^{113}\text{Cd}(\text{NO}_3)_2$ are shown in Figure 3d. The overall behaviour is very similar to what observed in KCl ; however, some of the spectral changes in KCl appeared at higher $\text{Cd}(\text{II})$ concentration likely because part of the $\text{Cd}(\text{II})$ is present in the CdCl^+ form. In both cases, G1N7 is the most affected resonance, followed by G2N7, G16N7 and G18N7 (Table S4). Interestingly, A22 shows a different behaviour in the two cases (Figure 3a and 3d). N1 resonance moved upfield by ~ 2 ppm in KClO_4 while in KCl moved by ~ 4 ppm and was nearly saturated at ~ 1 equiv. of added $\text{Cd}(\text{II})$. In both cases H2 chemical shift moved downfield by ~ 0.2 ppm. ^{113}Cd spectra at each point of the titration were collected in the two cases. Smooth upfield shift was observed in the titration performed in KClO_4 (Figure 4a), while the upfield shift observed until *ca* 1.5 mM of added $\text{Cd}(\text{II})$ in KCl was followed by a downfield shift (Figure 4b).

Figure 4: Stackplot of ^{113}Cd NMR spectra of a) 0.63 mM D1 ζ in the presence of KClO_4 titrated with increasing amounts of Cd(II) (pD 7.0, 300 K, 500 MHz), b) 0.64 mM D1 ζ in the presence of KCl titrated with increasing amounts of Cd(II) (pD 6.7, 300 K, 500 MHz), c) comparison of ^{113}Cd δ of D1 ζ and D1 $\kappa\zeta$, d) comparison of ^{113}Cd δ of D1 ζ in the presence of either KClO_4 or KCl , e) ^{113}Cd δ of D1 ζ in the presence of KCl with 4 linear intervals corresponding to different binding events.

^{31}P spectra to assess the binding

One of the most preferred metal ion binding sites is the negatively charged RNA phosphate backbone. Besides the well separated triphosphate signals of G1 (G1 $_{\text{TP}}$) (Figure S1, inset), all the other resonances are clustered around 0 ppm. In order to **assign** the strongly overlapped ^{31}P signals, $^1\text{H}, ^{31}\text{P}$ -HSQC-NOESY spectra [33] of D1 ζ were recorded. The experiment is based on the correlation of the backbone phosphates to aromatic and sugar protons of neighbouring residues. Correlations to H8/H6 and H1' were mainly used for the **assignment** and, even if ^{31}P spectra show quite broad peaks, some of them could be confidently **assigned** (Figure S1). After addition of 0.9 equiv. of Cd(II) , most of the resonances disappeared, and a few peaks underwent slight upfield shift, for example A6p and A21p resonances. Upfield shifts was also observed for G16p, A17p, C19p and U20p resonances at 6 equiv. of Cd(II) (^{31}P **assignments** refer to 5'-phosphate, see Figure S1). All three metal ions caused line broadening and consequent disappearance of ^{31}P resonances in 2D spectra. Therefore 1D ^{31}P NMR was used to monitor ^{31}P chemical shift changes upon addition of the different metal ions (Figure S2). The ^{31}P spectra show an overall generalized broadening upon metal ion addition. However, despite the strong overlap, a few peaks could be followed during the titration. For example, G2p and A22p resonances show significant changes, which is in agreement with spectral changes recorded by 2J - $^1\text{H}, ^{15}\text{N}$ -HSQC. Moreover, the more isolated peaks, U13p, C14p and G15p [34] only slightly moved and broadened, confirming the absence of localized interaction at the UUCG tetraloop. As far as the 5'-end phosphate is concerned, Cd(II) addition mainly caused broadening and shifting of the β -phosphate and γ -phosphate resonances, and only moderate line broadening of the α -phosphate signal, in line with direct coordination of Cd(II) to the β , γ -phosphates and N7 of G1 $_{\text{TP}}$ [35, 36]. The same behaviour was recently observed in the case of D1 $\kappa\zeta$ [12]. Similar broadening and shifting was observed with Mg(II) , even if less

pronounced, while only slight shifting was observed with cobalt(III)hexammine (data not shown). This behaviour confirms 2J - $[^1\text{H}, ^{15}\text{N}]$ -HSQC findings, which point to Cd(II) induced macrochelate formation.

Mn(II) titration

Mn(II) line broadening experiments are often employed to identify inner-sphere interactions [13, 14, 37]. We here recorded $[^1\text{H}, ^1\text{H}]$ -NOESY experiments in the presence of increasing amounts of Mn(II). At 30 μM of Mn(II) G1H8-H1' clearly disappears, confirming inner-sphere coordination at the 5'-end. At higher Mn(II) concentration cross peaks involving internal loop residues also start disappearing, *e.g.* A22H2-A6H2, A22H2-A6H1' and G23H8-A22H1' (data not shown).

Discussion

Comparison of the three metal ions

Mg(II) is considered the natural RNA cofactor. It can bind to RNA either via inner-sphere, *i.e.* direct coordination to oxygen or nitrogen atoms, or via outer-sphere interactions, where the coordination is mediated by water molecules (Figure 2). A number of metal ions are used to mimic Mg(II) binding. Cobalt(III)hexammine is used to mimic Mg(II) outer-sphere binding while Cd(II) is often the metal of choice to investigate inner-sphere binding. Here we followed ^1H and ^{15}N chemical shift changes in the presence of the three metal ions. **Interpreting chemical shift changes of large RNA upon metal ion addition is not trivial, since a combination of factors can influence the observed changes, like direct binding, close-by binding, or metal induced conformational changes. This may lead to not linear and not uniform chemical shift changes. The identification and separation of these factors is somehow elusive and here we will just qualitatively compare the differences observed among the various metal ions used.** Chemical shift changes ($\Delta\delta$) of H8, N7 and N9 in the three cases are summarized in Figure 5. All investigated metal ions **cause chemical shift changes on** G8 and G18, which are part of the ζ receptor. Interestingly, coordination to G8 (D1 ζ numbering) was also observed in the docked form of a similar tetraloop receptor (see above) [16]. **Cobalt(III)hexammine causes a significant $\Delta\delta$ of H8 with no shift of N7. Such a behaviour is**

compatible with either outer-sphere coordination or with close-by coordination, with subsequent conformational change [29]. On the contrary, the strong upfield shift of G18N7 upon Cd(II) binding suggests inner-sphere coordination in this case. The changes of G18 resonances upon interaction of with Mg(II) are similar as with Cd(II), but significantly weaker, which suggests either at least partial direct Mg(II) coordination at G18N7 but with much lower propensity than Cd(II), or coordination close-by. On the other hand, all metal ions influenced the chemical shift of G8N7 in a similar way and caused line broadening at 2.4 equiv. of Cd(II), 1.1 equiv. of cobalt(III)hexammine and 2.3 equiv. of Mg(II), suggesting a dynamic situation.

Figure 5: Comparison of H8, N7 and N9 chemical shift changes of D1 ζ in the presence of Cd(II), cobalt(III)hexammine and Mg(II) at the molar ratio $M^{n+}/\text{RNA}=2.9$. Red crosses indicate signals that disappeared during metal ion titration. Values in the presence of KCl are used for cobalt(III)hexammine and magnesium(II), and in the presence of KClO₄ for cadmium(II), see text.

It is clear that Cd(II) has the strongest effect in all instances, suggesting that inner-sphere binding is preferred over outer-sphere binding in this construct. Indeed, the upfield shift of N7 resonance is always accompanied by downfield shifts of N9 and H8 resonances in canonical helical regions (G1, G2, G16 and G18). The strong chemical shift changes observed at the G residues confirm the preference of Cd(II) for G-rich helical regions. Interestingly, there is a remarkable exception at A6 and A22 in the internal loop. A6H8 resonance moves upfield by ca 0.1 ppm in the presence of cobalt(III)hexamine, with a concomitant upfield shift of N9 and N7 resonances. On the other hand, Cd(II) causes only small downfield shifts of N9, N7, and H8 resonances, while the effect of Mg(II) on N9 and H8 resonances is similar, but slightly smaller to the one of cobalt(III)hexamine and almost no change is observed for N7 resonance. This behaviour suggests that there is no direct binding but only an electrostatic interaction at A6, with cobalt(III)hexamine having the strongest effect owing to its higher positive charge. Conversely, Cd(II) seems to have a bigger effect than Mg(II) and cobalt(III)hexamine on A22 N9 chemical shift, and this trend is consistent with the behaviour of N1 and N3 chemical shifts (see below).

Metal ion interaction with D1 ζ was detected on the changes in N1/N3/H2 chemical shifts as well. All three metal ions caused some shifting with subsequent line-broadening of the resonances of the internal loop residues A6 and A21, which supports assumption of predominant electrostatic interaction in the internal loop. However, patterns of chemical shift changes were somewhat different, probably partly influenced by outer- or inner-sphere coordination. For example, Mg(II) and cobalt(III)hexamine cause similar changes of A6N1/N3/H2 resonances, with cobalt(III)hexamine displaying the expected stronger effect (e.g. 0.3 equiv. of cobalt(III)hexamine are enough to induce equivalent changes caused by 1 equiv. of Mg(II)), while the Mg(II)-induced changes of A21N1/N3/H2 chemical shifts are similar to those of Cd(II). Differently, A22N1/N3/H2 resonances remain nearly unchanged in cobalt(III)hexamine but they shifted in Cd(II) and Mg(II). Since purine N1 sites in non-canonical regions are accessible for metal

ion coordination, we can speculate that A22N1 **may** represents a direct Cd(II) and Mg(II) binding site.

Metal ion binding in the three-way junction and in the isolated internal loop

Cd(II) and cobalt(III)hexammine interaction with D1κζ resulted in line broadening of the resonance of G45 from the GU wobble, which occupies the same position as G23 in D1ζ (Figures 1b, 1c and 3). This effect was attributed to either metal ion binding dynamics or homodimer formation involving the interaction between the GAAA tetraloop of one molecule and the ζ receptor of another [4, 12]. Since D1ζ contains only the internal loop and no GAAA tetraloop, the possibility of homodimer formation can be now excluded and the observed behaviour can be confidently attributed to metal ion binding dynamics at the GU wobble. Interestingly, cobalt(III)hexammine binding caused disappearance of G23N7 resonance followed by appearance of new peaks, which may suggest a dynamic situation in the molecule. In both constructs, with or without three-way junction, Cd(II) caused an upfield shift of G23N7 resonance (G45 in D1κζ, Figures 1b and 1c) accompanied by a downfield shifts of N9 and H8 resonances already in the first step of metal ion addition, which is typical for direct coordination (Figure 3a and Table S1) [12]. The subsequent line broadening of GN7 resonance may be attributed to the exchange of bound and unbound form on the intermediate time scale induced by inner-sphere coordination. Similarly, A6, A21 and A22 resonances (D1ζ numbering) have the same behaviour in both constructs, showing that the three-way junction does not strongly influence the metal ion coordination manners in the internal loop. However, the three-way junction strongly influences the metal ion binding at G40 (G18 in D1ζ, Figures 1b and 1c), whose N7 resonance was line-broadened in cobalt(III)hexammine and shifted upfield with subsequent line broadening in Cd(II) [12]. In D1ζ this guanine is positioned in a simple helical region, without facing the three-way junction, and its N7 resonance does not disappear upon metal ion addition. **In this case, direct inner-sphere interaction could be hypothesized for Cd(II) binding while more difficult is the interpretation of the changes observed with cobalt(III)hexammine (see above).**

As it was already reported for D1κζ, the Cd(II) signal detected in the ^{113}Cd NMR experiments represents an average signal of bound and free Cd(II), which with increasing amounts of added Cd(II) moves upfield towards to the position of the free Cd(II) (0.8 ppm) [12]. The position of the Cd(II) signal at the beginning of the titration is the same for D1ζ and D1κζ (~ 40 ppm, Figure 4c) and it is mainly due to macrochelate formation at the G1 triphosphate, which represents the strongest binding site. The ^{113}Cd chemical shift moves linearly in both RNA constructs towards the chemical shift position of free Cd(II) (Figure 4c). However, smaller chemical shift change was observed in D1κζ, suggesting a strong effect of the three-way junction on ^{113}Cd chemical shift and confirming that the three-way junction itself is an important Cd(II) binding site.

Comparison of Cd(II) binding in the presence of either KCl or KClO₄

Since monovalent metal ions are needed to stabilize RNA secondary and tertiary structure, RNA samples for NMR studies are commonly prepared in KCl solutions, likewise the published NMR solution structure of D1κζ [4]. Correspondingly, all the here reported experiments were performed in the presence of KCl. However, Cd(II) is known to form with chloride the CdCl^+ species, which also has the ability to interact with RNA. For this reason, we performed the Cd(II) titration in the presence of KClO_4 as well. The overall binding behaviour observed in the $^2J\text{-}[^1\text{H},^{15}\text{N}]\text{-HSQC}$ spectra was similar in the presence of KCl or KClO_4 , with only the A22N1 chemical shift change being more significant in the presence of KCl, suggesting that monovalent CdCl^+ ions may contribute to the binding to the non-canonical A22N1 (Figures 3a and 3d). This behaviour somehow resembles the observed monovalent metal ion binding site observed in the AA platform of the docked tetraloop receptor in the crystal structure of *Tetrahymena* P4–P6 domain [21]. Interestingly, this binding site seems to be preferred by monovalent CdCl^+ and divalent Cd(II) and Mg(II) ions over cobalt(III)hexammine. Similar behaviour was also observed in the case of the docked tetraloop, with monovalent and divalent metal ions being preferred over cobalt(III)hexammine in the coordination at the AA platform [20]. Conversely, the ^{113}Cd NMR spectra in the presence of KCl and KClO_4 differed greatly. On the one hand, the average ^{113}Cd signal of bound and free Cd(II)

in the presence of KClO_4 shows a linear shift to an upfield position, which is proportional to the increase in $\text{Cd}(\text{NO}_3)_2$ concentration (Figures 4a and 4d). On the other hand, the plot of the chemical shift variation of the ^{113}Cd signal in the presence of KCl shows 4 linear intervals with different slopes (Figures 4d and 4e). Comparison of the $^{113}\text{Cd}(\text{II})$ spectra (Figure 4b) with the $^2J-[^1\text{H}, ^{15}\text{N}]$ -HSQC spectra (Figure 3a) suggests that these intervals reflect different binding events within D1 ζ , and enables us to speculate the following: the steepest interval I (from 0.3 equiv. to 1.1 equiv. of $\text{Cd}(\text{II})$, Figure 4e) could be attributed to the macrochelate formation and to the coordination of $\text{CdCl}^+/\text{Cd}(\text{II})$ to A22N1. Intervals II and III (1.3-3.4 equiv. of $\text{Cd}(\text{II})$, Figure 4e) are dominated by chemical shift changes in G2N7/H8, G16N7/H8, A17N7/H8 and G18N7/H8. The slight decrease within interval III reflects the growing influence of CdCl^+ in the average chemical shift, which shows a downfield chemical shift with respect to $\text{Cd}(\text{II})$ [38]. Most of the strongest binding sites are already saturated within interval IV (3.4-5.3 equiv. of $\text{Cd}(\text{II})$, Figure 4e), where the observed ^{113}Cd downfield shift is mostly influenced by CdCl^+ resonance. In excess of $\text{Cd}(\text{II})$ (9 equiv. and 22 equiv. of $\text{Cd}(\text{II})$, data not shown) the ^{113}Cd chemical shift is constant at ~ 35 ppm.

Conclusions

In this work we used NMR to elucidate the metal ion binding properties of an RNA internal loop. Our NMR data confirmed the tendency of $\text{Cd}(\text{II})$ to coordinate to G-helical residues, the high prevalence of a $\text{Cd}(\text{II})$ induced macrochelate at the 5'-end triphosphate, and suggest an overall weak interaction of this RNA construct with cobalt(III)hexamine. **The overall small chemical shift changes experienced by nucleotides located in the internal loop suggest a general electrostatic interaction. However, the three metal ions show a slightly different behaviour: cobalt(III)hexamine addition mainly influences A6 resonances, while A22 resonances are the most affected upon addition of $\text{Cd}(\text{II})$ and $\text{Mg}(\text{II})$. Moreover, the behaviour of A22N1 resonance upon addition of $\text{Cd}(\text{II})$ in KCl suggests the presence of a monovalent CdCl^+ binding site.** Comparison of our data with previously published data on metal ion titrations of the RNA construct containing both the internal loop and the three-way junction confirmed the effectiveness of the

three-way junction as metal ion binding site. Moreover, ^{113}Cd NMR proved to be a powerful tool to investigate RNA binding events, being extremely sensitive to the nature of the Cd(II) containing species. Finally, significant differences were observed when Cd(II) additions were performed in the presence of either KCl or KClO_4 , and this should be taken into account when planning Cd(II) titrations. In conclusion, this work further explored the potential of heteronuclear NMR in drawing a detailed picture of metal ion binding to RNA molecules, and its ability to ascertain even subtle changes in the binding behaviour of different metal ions.

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Graphical Abstract

Metal ion binding to an RNA internal loop

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