Published in final edited form as:

J Mol Biol. 2007 September 7; 372(1): 89–102.

Structural metals in the group I intron: a ribozyme with a multiple metal ion core

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Summary

Metal ions play key roles in the folding and function for many structured RNAs, including group I introns. We determined the X-ray crystal structure of the *Azoarcus* bacterial group I intron in complex with its 5' and 3' exons. In addition to 222 nucleotides of RNA, the model includes eighteen Mg²⁺ and K⁺ ions. Five of the metals bind within 12 Å of the scissile phosphate and coordinate the majority of the oxygens biochemically implicated in conserved metal-RNA interactions. The metals are buried deep within the structure and form a multiple metal ion core that is critical to group I intron structure and function. Eight metal ions bind in other conserved regions of the intron structure, and the remaining five interact with peripheral structural elements. Each of the eighteen metals mediates tertiary interactions, facilitates local bends in the sugar-phosphate backbone or binds in the major groove of helices. The group I intron has a rich history of biochemical efforts aimed to identify RNA-metal ion interactions. The structural data are correlated to the biochemical results to further understand the role of metal ions in group I intron structure and function.

Keywords

group I intron; ribozyme; metal ions; heavy metal soaks; RNA structure

Introduction

Ribozymes and other complex structured RNAs require metal ions for proper folding and function^{1, 2}. To adopt a native conformation, folded RNAs pack helices and linker segments into a compact structure, resulting in the juxtaposition of poly-anionic sugar-phosphate backbones. Cations, such as Mg²⁺ ions, associate with RNA through two types of interactions. First, they bind diffusely, neutralizing the charged RNA backbone through multiple water layers. Second, a metal ion can shed bulk solvent and interact with RNA ligands directly or through its inner hydration shell³. RNA-metal contacts are critical to RNA form and function; disruption of a single RNA-metal interaction can inhibit RNA catalysis, even if the interaction is distant from the active site^{4, 5}.

X-ray crystallographic studies of metals bound to tRNAs, the P4-P6 domain of the group I intron, the SRP and rRNAs have complemented biochemical characterization of RNA metal binding motifs^{6; 7; 8; 9; 10; 11; 12; 13}. The major groove of A-form helices, tetraloops, tetraloop receptors, and irregularly shaped RNA backbones are common sites of metal binding

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in crystal structures. In the 50S *Haloarcula marismortui* ribosome crystal structure, where over 200 metals were identified, individual metal binding sites were analyzed and then classified according to the metal species and level of hydration¹³. Most Mg^{2+} ions and some monovalent ions interact directly with phosphate oxygens of the rRNA, while the majority of Na^+ and K^+ ions contact polar groups in the major groove of helices.

These findings are consistent with the interplay of charge density, desolvation energy, and preferred coordination geometry in determining metal binding specificity³. Mg^{2+} ions frequently coordinate charged phosphates directly because these strong electrostatic interactions compensate for a high desolvation penalty. K^+ ions, with a lower charge density and smaller dehydration energy, can directly interact with polar groups in the electronegative A-form helix major groove. If alternative metal binding changes the native RNA structure significantly, folding and/or activity of the RNA can be disrupted, leading to a structural requirement for a particular metal ion^{11; 14}.

Crystal structures including assigned ordered metals have been used in combination with biochemical and biophysical analysis to predict the roles of metal ions in RNA folding. Mg^{2+} dependent RNA folding and tertiary stabilization has been modeled in tRNAs and pseudoknots as entirely dependent on diffusely binding ions^{15; 16}. In the case of a more complex RNA domain where three helices pack against each other, tertiary structure formation has been described as folding around a 'core' of metal ions which interact with metal ligands biochemically demonstrated as important for folding⁹. Examples of crystallographically identified metal clusters are in the three helical junction in the P4-P6 domain of the *Tetrahymena* group I intron and protein-deficient regions of the ribosome^{9; 13}. Additional identification of metal ion clusters in complex RNAs will further establish trends in metal binding at multiple helical interfaces. Comparing crystallographic data with biochemical information can provide insight into the roles of these metals in folding and catalysis of RNAs^{5; 9; 17}.

The self-splicing group I intron is an attractive target for the structural examination of RNA-metal ion interactions because of an extensive biochemical analysis demonstrating metal ion involvement in folding and catalysis. In the presence of Mg^{2+} or Mn^{2+} and a guanosine cofactor, group I introns catalyze two phosphoryltransfer reactions resulting in ligated exons and an excised intron¹⁸. Specific RNA-metal ion interactions have been identified in the active site, demonstrating a role for metal ions in catalysis^{19; 20; 21; 22; 23; 24; 25}. In addition to the catalytic metal ion ligands examined, important metal-RNA contacts beyond the active site have been identified through thiophilic metal rescue of phosphorothioate interference, hydroxyl-radical footprinting, and metal dependent hydrolysis^{4; 5; 14; 17; 26; 27}. These studies have been performed on group I introns from several different organisms, with conserved regions having similar biochemical and structural properties^{27; 28; 29}. The group I intron studies described above have identified intron metal ligands and intron regions that require metals to fold. They do not, however, generally indicate which ligands are linked to the same metal, which tertiary interactions the metals are facilitating, or if multiple metals group together. X-ray crystal structures can be used to address these questions.

Four group I intron crystal structures have been reported. These include the *Azoarcus* (3.1 Å and 3.4 Å resolution), *Tetrahymena* (3.8 Å) and phage Twort (3.6 Å) group I introns from the IC3, IC1 and IA2 classes, respectively^{30; 31; 32; 33}. The two highest resolution structures capture the *Azoarcus* pre-tRNA^{Ile} group I intron and its flanking exons aligned for the second step of splicing^{30; 33}. The 3.1 Å resolution *Azoarcus* structure includes an inactivating 2'-deoxy substitution at the terminal nucleotide of the intron, ΩG, which removes a catalytic metal ion ligand^{20; 34}. Previous reports described the changes in active site metal binding due to the 2'-deoxy substitution, the overall fold of the ribozyme, the splice site selection machinery, and

the two-metal-ion phosphoryltransfer mechanism implied by the structures^{30; 33; 35; 36}. Here we report the identification and position of metal ions within the *Azoarcus* group I intron crystal structures. We then describe the roles of these metals in RNA splicing based on comparison to a diverse set of previously published biochemical data on metal involvement in intron function.

Results

Metal assignment

After the initial model of the *Azoarcus* pre-tRNA^{Ile} group I intron-two exon complex was built and refined into experimental electron density, non-nucleotide density remained in the $F_o - F_c$ and $2F_o - F_c$ difference Fourier maps. This solvent density was initially built with water molecules and considered for metal re-assignment after further positional and B-factor refinement.

Several crystallographic indicators were used to identify which non-nucleotide density was attributable to ordered Mg^{2+} and K^+ ions and which locations should remain as waters. First, refined solvent B-factors were evaluated for consistency with the B-factors of surrounding nucleotides. A vastly lower temperature factor for a water molecule was indicative of additional electrons, such as would arise from a K^+ ion. Second, the coordination geometry of each putative metal was considered. Mg^{2+} ions have inner sphere ligands in an octahedral geometry, while K^+ ions typically bind with irregular ligand orientation^{37; 38}. Third, the proximity of a solvent molecule to potential ligands was analyzed. Mg^{2+} ions bind with an ideal ligand distance of 2.0-2.4 Å, whereas K^+ and water interactions to polar groups are longer (2.8-3.5 Å)^{37; 38}. Finally, the putative K^+ or Mg^{2+} position was compared with binding sites of heavy metals detectable by anomalous differences in the diffraction data (see below). Using these four criteria, 18 out of 189 solvent atoms in the structure were assigned as metal ions.

A series of metal salts were soaked into the deoxy-ΩG group I intron crystals to serve as heavy metal mimics for the identification of metal binding sites. The soaked compounds included $YbCl_3$, $TbCl_3$, $EuCl_3$, $MnCl_2$, $ZnCl_2$, Na_3IrCl_6 , $Co(NH_3)_6$, $TiOAc$ and $RbCl$. Data were collected at a wavelength where a large anomalous signal from the soaked metal could be detected (Table 1). $ZnCl_2$, Na_3IrCl_6 , $Co(NH_3)_6$ and $RbCl$ soaked crystals yielded data sets with no significant anomalous signal. However, anomalous difference peaks, some as big as 30σ , were observed in data collected from $YbCl_3$, $TbCl_3$, $EuCl_3$, $MnCl_2$, and $TiOAc$ soaked crystals (Table 1, Fig. 2). Though the resolution of these data were modest (3.7 Å-5.5 Å) (Table 1), detection of anomalous signal combined with rigid body refinement of the original coordinates facilitated the location of metal binding and correlation with native electron density. Each anomalous peak that agreed with native solvent density was assigned to a full occupancy metal ion. Several sites of heavy metal binding did not have corresponding native density and metals were not assigned at these locations. This disparity could arise from differential affinity between the mimics and native metals for local RNA substructures.

In the deoxy-ΩG structure, ten assigned metal ions were supported by at least one anomalous difference peak from a Mg^{2+} or K^+ mimic (Table 1, Fig. 2). All five K^+ ions (M2, M4-7) correspond with a Tl^+ peak and each had an unusually low temperature factor when assigned as a water or Mg^{2+} ion. Five Mg^{2+} ions (M1, M3, M11, M12, M14) share binding sites with at least one lanthanide. Eight additional Mg^{2+} ions (M8-M10, M13, M15-M18) were modeled into the deoxy-ΩG native density based on proximity and relative orientation of phosphate oxygens to the metal ion. In total, 18 Mg^{2+} and K^+ ions were assigned in this structure (Fig. 1).

TbCl₃, YbCl₃, MnCl₂, and TIOAc soaks were repeated with the ribose-ΩG construct. The ribose-ΩG crystal soaks generally agree with the deoxy-ΩG soaks, with one major exception. The metal binding patterns differed near the ΩG 2'-OH, resulting in both M1 and M2 being Mg²⁺ ions³³. The ribose-ΩG crystallization construct is an active form of the ribozyme^{33, 35}, therefore the position and identity of these two active site metals (M1, M2) are most relevant for consideration of the reaction mechanism and native active site structure. Outside of the active site, the overall intron architecture is equivalent and three of four ribo-ΩG metal locations match those in the deoxy-ΩG structure. As a result, the 16 non-active site metals assigned in the higher resolution deoxy-ΩG structure were used to characterize the non-active site RNA-metal ion interactions identified.

Surface potentials of the group I intron

We next asked if the ordered metal ions bound to areas of lowest electrostatic potential within the structure. Electrostatic potentials were calculated and mapped onto a surface representation of the crystal structure (Fig. 3). The lowest potentials lie on active site residues, closely packed phosphates and a few non-canonical major groove surfaces. The four most negative surfaces (-115 kT/e to -90 kT/e) are on exposed active site residues, with M1 and M2 bound underneath or next to the surface. The next most negative potentials (-90 kT/e to -85 kT/e) are found on J6/7, where M5 binds, and in the L5 tetraloop. The P2 tetraloop surface has a similar location of low potential (< -65 kT/e). Juxtaposed phosphates account for the next lowest group of surfaces (-65 kT/e to -60 kT/e). These locations include the packing surface of P2 and P8 as well as the J8/7-J6/7 interface. These are the location of M18 and M12 binding, respectively. Major groove surface potentials are also found in this range over the bases of nucleotides 65-66, where M10 is bound, and 58-59, near M11. Five surfaces of the structure have lowest potentials of -55 kT/e to -50 kT/e. These include the phosphate rich binding sites of M3, M16, M17 and M18 as well as the P8 tetraloop receptor, which binds M4. Additional negative potential peaks (-50 kT/e to -45 kT/e) are observed at phosphate junctions near M13, M8 and two other interfaces.

The remaining negative surface potentials highlight general features of folded RNA. From -40 kT/e to -35 kT/e, several peaks near phosphate bends appear, including those near M14 and M15. Many major groove surfaces have a lowest potential between -35 kT/e and -25 kT/e, including the binding sites for M6, M7 and M9. Finally, each phosphate surface has a potential lower than -20 kT/e.

Out of the 23 most negative surface potentials located on the intron (< -45 kT/e), only five do not have an associated crystallographically assigned metal. These exceptionally negative surface potentials corroborate 13 of 18 metals assigned, with the remaining 5 bound to negative surfaces in well-characterized RNA-metal binding motifs. These data suggest that the metals identified in the crystal structure could bind favorably in solution by compensating the partial dehydration energetic penalty with strong electrostatic interactions.

Discussion

The location of ordered metal ions in RNA crystal structures can provide insight into how metals are bound to neutralize phosphate rich RNA folds. When combined with biochemical analysis, the crystallographically identified metals can be associated with a functional role in folding, structural stabilization or catalysis. Extensive metal binding assays and the recent crystal structures of the group I intron provide a platform for such analysis.

Four crystal structures of the group I intron have been reported at a resolution sufficient to observe metal ions^{30; 31; 32; 33}. The *Tetrahymena* (3.8 Å resolution) and Twort (3.6 Å resolution) structures each include only one metal in the active site and several Mg²⁺ ions in

the remainder of the intron^{31; 32}. The Twort non-active site metals are a subset of the 18 *Azoarcus* metals, overlapping with M3, M5 and M16. The *Tetrahymena* metals, assigned solely from Eu³⁺ and Ir³⁺ soaks³¹, do not overlap significantly with the *Azoarcus* metals. The differences in number and location of metal ions in the group I intron crystal structures may result from increased difficulty of metal assignment at lower resolution or from binding differences between Mg²⁺ and Eu³⁺ and Ir³⁺. Since the backbones of all the group I crystal structures superimpose in conserved structural elements^{28; 39}, the metal binding sites in these regions of the *Azoarcus* structure are expected to reflect those in other group I introns.

Multiple metal ion core

Within the *Azoarcus* intron structure, five of the crystallographically observed metal ions (M1-M3, M5, M12) bind in the heart of the ribozyme, and coordinate the majority of the oxygens biochemically implicated in conserved metal-RNA interactions (Fig. 4, Table 2). The metals are buried deep within the structure and make multiple inner sphere contacts to phosphate and sugar oxygens. These five metals constitute a metal ion core, each bound within 12 Å of the scissile phosphate and positioned to screen phosphate charge, align nucleotides from different structural elements and/or catalyze phosphoryl transfer.

The catalytic metal ions, M1 and M2, are important not only for activation of substrates and stabilization of the transition state^{19; 20; 21; 22; 23; 40}, but also for formation of the active site structure¹⁴. The group I intron active site is composed of phosphates and sugars from three intron joiner regions (J6/7, J4/5 and J8/7) and the three substrate nucleotides (A+1, U-1 and ΩG). The result is a highly charged active site, with six intron phosphates positioned within 6 Å of the scissile phosphate. The two catalytic Mg²⁺ ions (M1, M2) bridge each of these components through ten inner sphere contacts: five to substrate oxygens and five to the surrounding intron active site pocket (Fig. 4a). A sixth water ligand is assumed for M1 and M2, but is not visible in the structure. Disruption of all but one of the ten direct RNA contacts through sulfur or amino substitution results in strong inhibition of splicing activity, with seven of the effects rescued by thiophilic metal ions (Table 2). Three phosphates (A+1, A172, G128) coordinated by M1 and M2 are also sites of Tb³⁺ dependent hydrolysis¹⁴, suggesting that two metals are responsible for the three cleavage events.

M1 and M2 bind to nucleotides involved in splice site selection. Proper 3'-splice site selection is achieved through hydrogen bonds and stacking interactions between the ΩG and P7 and J6/7^{30; 41}, while 5'-splice selection depends on interactions between the G•U-1 wobble pair and sheared A-A pairs in J4/5⁴². Three of the intron nucleotides involved in splice site selection are coordinated by M1 and M2 (Fig. 4a). The metal-oxygen interactions with the phosphates of A87 and C88 in J4/5 flank the A87 base, which makes critical hydrogen bonds to the wobble pair at the 5'-splice site. Additionally, M2 coordinates the G128 phosphate between two bases that stack above and below the ΩG, part of the J6/7-P7 G binding site responsible for 3'-splice site selection.

The Mg²⁺ dependent folding event that occurs in the group I intron active site may be attributable to the M2 binding observed in this structure. After folding in Na⁺ ions, addition of Mg²⁺ induces a change in the hydroxyl radical footprinting pattern¹⁴, which is localized to J6/7 nucleotides G128-G130 in the G binding site loop. The Mg²⁺ induced protection at these nucleotides correlates with intron activity, consistent with a metal binding change near the active site¹⁴. M2 and the J6/7 strand, which interact at the G128 phosphate, are closer to the scissile phosphate in the Mg²⁺ bound ribose-ΩG structure than the K⁺ bound deoxy-ΩG structure^{25; 33; 35}. These data are consistent with an altered M2 binding site that accommodates monovalent cations at high concentrations and with Mg²⁺ binding organizing an active conformation that brings J6/7 towards the scissile phosphate.

M5 and M12 mediate the packing of J6/7 against J8/7 (Fig. 4b). The C171 phosphate in J8/7 is positioned adjacent to the backbone of U124-A127. M5 and M12 are only 3.9 Å apart and bridge between these two backbones by binding a phosphate oxygen ligand on each strand. M5 coordinates C171 in J8/7 and U124 and A127 in J6/7, while M12 binds the same oxygen ligands in C171 and U124. As indicated by phosphorothioate interference at U124 and C171, and by a metal specificity switch at A127 (Table 2), these metal-RNA interactions are important for activity even though they are not directly in the active site^{24; 25; 27}.

The five core metals (M1-M3, M5, M12) facilitate an M-shaped backbone conformation in J8/7 (Fig. 4d). J8/7 positions its nucleotides to make hydrogen bonds and stacking interactions with disparate regions of the ribozyme. The M shaped portion of J8/7 involves nucleotides G169 through U173 (in P7) with the points on the M corresponding to the five phosphates of these residues. The active site metals, M1 and M2, bind the peaks of the M at the phosphates of G170 and A172. M1 bridges between the *pro*-S_P oxygens of these two phosphates, while M2 binds the *pro*-R_P oxygen of A172. In the valley of the M, the C171 *pro*-R_P phosphate oxygen is directed away from the active site and is contacted by both M5 and M12. The end of the M is the U173 phosphate, which is the first nucleotide in P7. The transition from J8/7 into P7 includes a tight twist of the backbone where M3 binds with inner sphere coordination to two consecutive phosphates that both show phosphorothioate interference (Fig. 4c-d)²⁷.

P4-P6 metal binding

The P4-P6 helical stack provides a structural scaffold for the group I intron, as the other two helical domains pack into its major and minor grooves³⁵. The structural metal ions make extensive interactions with this domain and in several cases bridge between P4-P6 and other structural elements (Fig. 1b). As described above, four of the five core metals (M1-M2, M5 and M12) make direct contacts to P4-P6. Seven additional metals (M8-M11, M14-M16) also bind in this domain (Table 3).

M8 and M16 bind at the J6/7-P4 triple helical interface (Fig. 5a). The first two residues of J6/7 anchor the G binding motif into P4 through base triple interactions. J6/7 enters and exits this triple helix within three nucleotides, inserting the phosphate of U126 into the major groove of P4. The U shaped backbone in this region is bound by M5 and M12 on the J8/7 face, as described above, and by M8 and M16 on the P4 major groove face. M8 coordinates the bulged U126 phosphate and makes outer sphere contacts to consecutive GC pairs in P4. M16 binds at the site of J6/7 and J3/4 strand exchange from the P4-P6 helical stack. The metal ion lies between the phosphates of the first nucleotide of J6/7 and the first nucleotide of P4. This Mg²⁺ ion makes one inner sphere and several water mediated contacts to phosphates and 2'-OHs of the two strands. A third metal in this region, M14, binds at the interface of J3/4 and P4 between the close parallel phosphate backbones of P7 and J3/4 (Fig. 5b).

M16 is biochemically supported by a single site of Tb³⁺ cleavage in P4 at U51¹⁴ and neighbors a *pro*-R_P oxygen (A131) that shows Mn²⁺ rescue of phosphorothioate interference²⁵ (Table 3; Fig. 8b). The two inner sphere contacts made by M16 and M8 are to *pro*-S_P oxygens. The M16 ligand at G125 shows mild phosphorothioate interference²⁷, while the M8 ligand has not been tested. M14 binds directly to two *pro*-R_P oxygens that do not show phosphorothioate interference of activity in *Azoarcus*²⁷. One M14 ligand (A48) does show strong folding interference in the equivalent position in the *Anabaena* group I intron²⁹, suggesting the M14-RNA interactions visualized in the structure are important for folding.

Four additional metals (M9-M11, M15) make major groove interactions to J4/5 and P5. M11 and M9 are bound in the major groove of P5 and J4/5, opposite minor groove interactions with P10, P1 and the P9 tetraloop (Fig 4c). M11 is bound deep in the major groove, while M9 binds the shallow major groove of the P5 tetraloop receptor. M10 binds to two consecutive GC pairs

in P5 and a final metal ion binds to P4-P6 in the peripheral L5 tetraloop (Fig 4d). This Mg^{2+} ion (M15) coordinates two loop phosphates and makes major groove interactions with the base pair flanking the tetraloop. The *pro-R_p* phosphate oxygens within outer sphere interaction distance of these metals do not show phosphorothioate interference²⁷. However, a single Tb^{3+} cleavage supports M9 binding near A81 in P5¹⁴.

P10-P2 and P8 TLR metal binding

Five metal ions (M4, M6, M7, M13, M18) are bound within the P10-P1-P2 helix and its tetraloop receptor in P8 (Table 3). One metal is bound in the helical groove opposite the 5'-splice site, while the remaining four bind peripheral elements not conserved across group I intron classes.

The GU pair at the 5'-splice site binds a K^+ ion (M6) in its major groove (Fig. 6a). While the minor groove of this pair is specifically selected by hydrogen bonds with the J4/5 wobble receptor⁴², the deep major groove of the exon-intron helix is neutralized by this cation. The K^+ ion directly coordinates both the O6 of G10 and the O4 of U-1. The flanking AU pairs provide additional O4 and N6 ligands (Table 3). This sequence has been implicated in manganese binding by NMR, implying a divalent ion can bind the major groove in the absence of K^+ ions⁴³.

M13 binds two consecutive phosphates in the junction between helix 2 and 3 (Fig. 6b). M13 contacts the first two residues in J2/3, which are in a condensed backbone conformation that allows J2/3 to make tertiary contacts with the minor groove of P2. This metal binding site was biochemically predicted in the *Azoarcus* group I intron. It neighbors a site of Tb^{3+} cleavage¹⁴, and the G38 and A39 *pro-R_p* phosphate oxygens, which show strong phosphorothioate splicing interference²⁷, presumably due to disruption of the P1 substrate helix docking into the active site. This interference is not seen in the *Tetrahymena*²⁷ or *Anabaena* introns²⁹, possibly reflecting differences in structure and/or metal binding at the junction of P8, J8/7 and P2 among these group I introns.

The final three metals in this domain facilitate packing of P2 against P8 in the *Azoarcus* group I intron. M18 binds at the juxtaposition of P2 and P8 sugar phosphate backbones (Fig. 6c). P2 and the L2 tetraloop bind a K^+ ion (M7) in the major groove parallel to the final GC pair of P2 (Fig. 6c). The first G of the tetraloop stacks under this GC pair and its O6 is coordinated by the K^+ ion. These metals were not biochemically predicted. Finally, a K^+ ion (M4) binds in the P8 tetraloop receptor, a well characterized structural motif and monovalent metal ion binding site⁵. It directly coordinates the N7 and O6 of the GU pair below the A-A platform on which the P2 tetraloop stacks (Fig 5d). The metal ion is positioned to coordinate a phosphate and an adjacent O2' of the two juxtaposed As in the platform. This K^+ has ligands identical to the K^+ bound in a tetraloop receptor described in the P4-P6 domain of the *Tetrahymena* ribozyme⁹, and the functional importance of the K^+ is corroborated biochemically by Tl^+ rescue of sulfur substitution interference at G151 O6⁵.

P9.0/9 metal binding

J9/9.0 includes a reverse kink turn, which is a 90° asymmetric bend toward the major groove between a canonical (P9) and non-canonical (P9.0) helical segment³⁶. In this type of turn, the longer strand is on the inside of the bend and the backbone of the non-canonical helix packs into the major groove of the canonical helix. The J9/9.0 reverse kink turn binds a Mg^{2+} ion (M17) on the inside strand at the site of helical packing (Fig. 7). M17 makes one inner sphere and two outer sphere contacts to 3 of 4 consecutive phosphates (G181, G182, G184) (Table 3). The A183 phosphate of this strand bulges in the opposite direction of the turn, and is not accessible to this metal. The metal also makes outer sphere contacts to the base of G184, thereby

mediating helical packing. The turn of P9.0/P9 appears to be structurally conserved across intron classes^{31; 32; 35}. Although single sulfur substitution of these ligands does not inhibit activity²⁷, metal neutralization of this sharp bend would be expected in other examples of this turn.

Biochemical and structural correlation

The vast majority of structural and biochemical data on group I intron metal ligands are in agreement. Crystallographically identified metals account for 10 of 11 ligands that display a metal specificity switch upon sulfur for oxygen substitution (Fig. 8). This strong correlation between crystallographic and solution studies is possible because of the extensive biochemical analysis of metal binding in this RNA, including many semi-synthetic experiments that were required to examine *pro*-Sp phosphate oxygen ligands (Table 4). Ordered metals observed within the structure suggest that 11 phosphorothioate effects that did not display thiophilic metal rescue can also be attributed to metal binding (Table 4, Fig. 8). Lack of metal specificity switch of some structurally identified ligands may be due to differences in experimental conditions, but could also reflect the specificity of the binding sites for a metal ion of particular size, charge density or ligand geometry. The structure has provided additional information about metal binding where biochemical experiments have not attempted. Three previously unsubstituted *pro*-Sp oxygens at U15, U126 and G181 are predicted to be metal ligands (Table 3).

Several RNA-metal ion interactions identified outside of the metal core do not influence splicing (Table 3). Of seven non-core phosphate ligands substituted with sulfur in biochemical studies, four did not show phosphorothioate interference and the remaining three interferences could not be rescued with thiophilic metal ions (Table 3)²⁷. This is in contrast to the core metals, where disruption of each of the 14 metal-phosphate interactions results in inhibition of activity and five of these effects could be rescued with thiophilic metal ions. This difference may indicate that the crystallographic metals not biochemically identified play a role solely in non-specific charge neutralization. Alternatively, there may be a requirement for more than one RNA-metal interaction to be disrupted before an effect on activity is observed.

Properties of metal binding sites

The metals can be categorized based on position in the structure. Six metals (M1, M2, M5, M12, M14, M18) bridge two or more secondary structure elements, and connect disparate parts of the intron by mediating tertiary interactions (Fig. 1b). Six metals (M3, M8, M13, M15-M17) interact with unusual bends in the backbone by directly coordinating phosphate oxygens. Eight metals (M4, M6-M11, M15) bind the electronegative major groove and nearby phosphates.

Seven of the major groove metal binding sites (all but M9) contain a central GU or GC pair. In each case, the metal is in the plane of the base pair, and flanking bases provide additional major groove metal ligands. While the major groove RNA binding sites are similar for K⁺ and Mg²⁺, the mode of interaction is different. K⁺ ions directly interact with O6 and other base functional groups, while Mg²⁺ ions make water mediated contacts.

The Mg²⁺ and K⁺ ions visualized in the structure show different trends in the number and type of interactions made with the RNA. Most inner sphere Mg²⁺ ligands are phosphate oxygens, while the outer sphere ligands are a mixture of phosphates and polar functional groups. K⁺ ions make direct contacts to both functional groups in the major groove and phosphate oxygens of unique RNA folds. Generally, the Mg²⁺ ions have 1-3 inner sphere RNA ligands, while four of the five K⁺ ions identified have four or more RNA ligands each. This trend may reflect the amount of coordination necessary for metal ordering in the crystal lattice as the properties of many disordered metals remain hidden. Both of the active site Mg²⁺ ions in the ribose-ΩG

structure have five inner sphere RNA ligands, which is quite unusual. There is only one metal ion with this level of coordination among the 116 Mg^{2+} ions assigned in the ribosome crystal structure¹³. To have two metals with this extent of coordination positioned so closely together is unprecedented in RNA structures determined to date.

Conclusions

The *Azoarcus* group I intron crystal structure provides a view of 18 metal ions that play an integral role in forming the native structure of the ribozyme. The cluster of metal ions at the heart of the intron coordinate buried phosphates in the active site, at sites of helix packing and in conserved idiosyncratic strand bends. When examined in the context of biochemical studies, this structure provides an example of a metal ion core critical to the structure and activity of a ribozyme.

Materials and Methods

Soaking/Stabilization

UP62 group I intron RNA was prepared and crystallized as described previously^{30; 33}. Harvested crystals were placed in a stabilization solution of 30% MPD, 10 mM $Mg(OAc)_2$ or $MgCl_2$, and 10 mM KCl at 25° C. Fresh stabilization buffer with soaking compound was added. Crystals were washed in this solution at least three times to remove crystal debris from the drop. Soaking conditions were 2 mM $MnCl_2$ overnight; 0.5 mM $TbCl_3$ overnight, 20 mM TIOAc 2 hrs, 2 mM TIOAc overnight, 0.5 mM $YbCl_3$ overnight, 0.1 mM $EuCl_3$ 2 hrs. Soaks attempted with greater than 0.2 mM $Co(NH_3)_6$, 0.5 mM $TbCl_3$ or 2 mM $MnCl_2$ shattered crystals and destroyed diffraction.

Freezing/Data collection

Crystals were flash frozen in liquid N_2 at -70° C. Data were collected at beamlines X25 and X29 at the National Synchrotron Light Source.

Data processing

Indexing, integration and scaling was performed using HKL2000⁴⁴. Anomalous difference Fourier maps were generated in CNS⁴⁵ using phases from the original deoxy-ΩG structure³⁰. Rigid body refinement was performed with REFMAC⁴⁶. Anomalous difference maps were superimposed onto the corresponding refined coordinates. Figures were prepared in Ribbons⁴⁷ and Pymol⁴⁸

Electrostatic potential calculations

Electrostatic potentials were calculated with the nonlinear Poisson-Boltzmann equation in the program APBS⁴⁹. AMBER charges and radii, ionic strength of 0.145 M, solvent radius of 1.4 Å, solute dielectric of 2 and solvent dielectric of 80 were used. The group I intron coordinate file was prepared for APBS with pdb2pqr⁵⁰. Potentials were visualized on the surface of the intron structure with the APBS plugin in Pymol⁴⁸

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank Jesse C. Cochrane and Sarah V. Lipchock for helpful comments on the manuscript, and members of the Strobel lab for suggestions on figure design. Supported by NIH grant P01 GM022778.

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Figure 1. Metals in the *Azoarcus* group I intron crystal structure

a: Ribbon diagram of the *Azoarcus* group I intron pre-2S deoxy-ΩG (PDB 1U6B) crystal structure with metals indicated as spheres. Metal numbering indicated are used throughout the text. Metal sphere color indicates binding region: core metals are orange; P10-P2, P8 metals are blue; P4-P6 metals are purple; the P9.0-9 metal is green. b. Secondary structure of the *Azoarcus* pre-tRNA crystallization construct with inner sphere metal ion coordination to sugar and phosphate oxygens indicated with black lines. Dashed lines indicate interactions with metals that bridge secondary structure elements. Solid black lines indicate coordination of phosphate on a single strand. Dash-dot-dash lines indicate direct major groove interactions. Inset: M2 coordination from ribo-ΩG structure (PDB 1ZZN).

Figure 2. Anomalous difference maps

Superposition of anomalous difference maps (purple mesh) on rigid body refined group I intron coordinates. The maps are contoured at 3.5 σ . The exons are shown in red and the intron is shown in grey.

Figure 3. Surface potentials of the *Azoarcus* group I intron

Color gradient from red (≤ -35 kT/e) to blue (≥ 35 kT/e). Metals, where visible, shown in black spheres. a. Arrow indicating active site, where M1 and M2 are buried beneath the surface. b. Side view of P7-P4-J6/7 interface. c. Back view of intron.

Figure 4. Metal ion core

Color-coding of the RNA segments is as in Figure 1. Inner sphere metal coordination is shown by dashed lines. Phosphate oxygens which show activity effect upon substitution are shown in black. Haloed oxygens are those where the effect could be rescued with thiophilic metal ions. Panel a is from PDB 1ZZN and panel b-d is from 1U6B. See text for discussion. a. M1 and M2 b. M12 and M5. c. M3 d. Metal binding to the M shaped J8/7 region. Non-bridging phosphate oxygens of J8/7 and P7 are shown. Inner sphere coordination of K^+ and Mg^{2+} ions are indicated with dashed lines.

Figure 5. P4-P6 bound metal ions

Interference data indicated as in Fig. 4. a. M8 and M16. b. M14. c. M11 and M9. d. M15 and M10. Several nucleotides in P5 and L5 are omitted for clarity.

Figure 6. P10-P2, P8 bound metal ions
Interference data as indicated in Fig. 4. a. M6, b. M13, c. M7 and M18, d. M4

Figure 7. P9.0-9 bound metal M17

Possible outer sphere M17 ligands discussed in text are highlighted in yellow.

Figure 8. Structural map of interference data

Effects of phosphorothioate and sugar oxygen substitutions summarized in Table 2-3 are mapped onto the crystal structure. Sulfur or amino substitution sites that show interference are depicted as small spheres on the crystal structure. Black spheres indicate interference but no rescue. Red spheres indicate that the substitution was rescued by the addition of a thiophilic metal ion. b. deoxy-ΩG (1U6B) metals near the active site are shown along with the biochemical mapping described in a. Mg^{2+} ions assigned in the structure in orange, K^{+} ions in blue. The ΩG O2' sphere is not displayed because it is absent in the structure. c. ribo-ΩG (1ZZN) metal ion core metals are displayed along with biochemical mapping described in a.

Table 1

Anomalous peak locations

Anomalous peaks larger than 4 σ are listed for each metal binding site

		Yb ³⁺ 0.5 mM 4.6 Å	Tb ³⁺ 0.5 mM 5.0 Å	Eu ³⁺ 0.1 mM 5.5 Å	Mn ²⁺ 2 mM 4.8 Å	Tl ⁺ 2 mM 3.7 Å	Tl ⁺ 20 mM 4.0 Å
M1	Mg ²⁺	29.3	12.9	8.5	7.1		
M2	K ⁺		5.2			30.5	25.3
M3	Mg ²⁺		8.4				
M4	K ⁺					32.0	26.2
M5	K ⁺					19.8	20.2
M6	K ⁺						6.6
M7	K ⁺					21.1*	21.3*
M11	Mg ²⁺		5.1	4.8			
M12	Mg ²⁺		6.8				
M14	Mg ²⁺			4.3			

* indicates that the heavy metal binds in a neighboring position to the 2F₀-Fc density.

Table 2

Core metal ligands

Native metals and their inner sphere ligands are listed along with interference assay data. Potential ligands under 3.0 Å for Mg²⁺ and 3.8 Å for K⁺ are listed. Inhibition column indicates if sulfur or nitrogen substitution of the oxygen ligand caused interference. Shaded regions indicate interference was rescued by addition of thiophilic metal ion addition. This is a biochemical signature suggestive of inner sphere metal ion coordination. Rate effect column indicates the rate decrease upon sulfur or amino substitution. Interference column indicates interference of polymerase incorporated phosphorothioates

metal	region	Azo	Tet	atom	dist (Å)	inhibition rescue	reference	rate effect	interference Azo, Tet	Tp ³⁺ [14]
M1	J8/7	A172	A306	pro-Sp	2.1	y	[24]	25-50		
	J5/4	C88	C208	pro-Sp	2.3	y	[24]	25-50		
	3'-exon	A+1	A+1	pro-Rp	2.3	y	[22,19]	1000		y
	5'-exon	dT-1	U-1	O3'	2.4	y	[19]	1000		
	J8/7	G170	A304	pro-Sp	2.5	y	[24]	2-5		
M2 (rOG)	3'-exon	A+1	A+1	pro-Rp	1.9	y	[22,19]	1000		y
	J6/7	G128	A262	pro-Sp	1.6	y	[25]	>10		y
	J8/7	A172	A306	pro-Rp	2.0	y	[26,27,24]	25-50	6,6	y
		G206	G414	O2'	2.1	y	[20]	~30		
		G206	G414	O3'	2.5	y	[21]	inactive		
	J5/4	A87	A207	pro-Sp	3.7	y	[24]	25-50		
M3	P7	U173	U307	pro-Rp	2.3	y	[27,26]		6,3	
		A174	A308	pro-Rp	2.1	y	[27,26]		6,2*	
M5	J8/7	C171	U305	pro-Rp	2.8	y	[27,26,24]	2-5	6,6	
	J6/7	U126	C260	O2'	3.0	n	[27,26]			
	P6	U124	U258	pro-Sp	3.3	y	[25]	5-10		
	J6/7	A127	A261	pro-Rp	3.4	y	[27,26]		3,0*	
M12	P6	U124	U258	pro-Sp	2.1	y	[25]	5-10		
	J8/7	C171	U305	pro-Rp	2.3	y	[27,26]	2-5	6,6	

* indicates data set with soft metal rescue where data sets differ. Potential outer sphere M2 ligand is shown in italics.

Metal ligands outside the core

Table 3

metal	region	Azo	Tet	atom	dist (Å)	inhibition rescue	reference	rate effect	interference Azo, Tet, Ana	Tb ³⁺ [¹⁴]
P4-P6 metals										
M8	J6/7	U126	C260	pro-S _p	1.8					
M9	P5	W162			2.0					
M10	P5									
M11	J5/4									
M14	P3	A48	C102	pro-R _p	2.6	n	[^{27,26,29}]		0,0,2	
	J3/4	U133	U267	pro-R _p	2.8	n	[^{27,26}]			
		W135			3.0					
M15	L5	C74		pro-R _p	2.3	n	[²⁷]			
M16		W164			1.8					
		W146			2.6					
	P6	G125	U259	pro-S _p	2.7	y	[²⁵]	2-5		
P1-P2, P8 metals										
M4	P8 (TLR)	A150		pro-R _p	2.5	n	[^{27,26}]			
		A149		O2'	2.7	n	[^{27,26}]			
				N7	2.9					
		G152		O6	3.0	n	[⁵]			
		G151		O6	3.4	y	[⁵]		10	
M6	P10	U9	U21	O4	2.8					
	5'-exon P1	A-2	A-2	N6	3.0					
		dT-1	U-1	O4	3.0					
	P1	W104			3.1					
M7		G10	G22	O6	3.2	n	[⁵]			
	L2	G24		O6	3.0	n	[⁵]			
		G23		O6	3.4	n	[⁵]			
M13	J2/3	G38		pro-R _p	1.8	y	[²⁷]		6	
		A39		pro-R _p	2.4	y	[²⁷]		6	
		G37		O2'	2.9	y	[²⁷]		3	
M18	P2	U15		pro-S _p	2.2					
P9,0-9 metal										
M17	P9.0	G181		pro-S _p	2.6					

The data are presented as in Table 2.

Table 4

Metal ligand frequency

The number of ligands identified in each category is listed

Inner Sphere Ligands	Mg ²⁺	K ⁺	total	substituted	interf	thiophilic rescue
pro-S _p	7	3	10	7	7	2
pro-R _p	9	4	13	13	9	2
O6		5	5	5	1	1
O2'	1	3	4	4*	1*	
O3'	1		1	1	1	1
O4		2	2	0		
N6		1	1	0		
N7		1	1	0		
O5'		1	1	0		
%phosphate	88.9	35.0	60.5			
Outer Sphere Ligands				S/N sub	interf	rescue
pro-S _p	Mg ²⁺	K ⁺	total	1	1	0
pro-R _p	14		14	14	4	4
O2'	13	1	14	7*	2*	
O6	6	1	7	6	0	
N6	1	1	2	0		
O4	2		2	0		
N7	3	2	5	0		
N4	1	1	2	0		
% phosphate	58.7	16.7	53.8			

* indicates deoxy substitution.