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Specificity of Mg^{2+} binding at the Group II intron branch site

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ABSTRACT

Metal ions play a crucial role in the conformation and splicing activity of Group II introns. Results from 2-aminopurine fluorescence and solution NMR studies suggest that metal ion binding within the branch site region of native D6 of the Group II intron is specific for alkaline earth metal ions and involves inner sphere coordination. Although Mg^{2+} and Ca^{2+} still bind to a mutant stem loop sequence from which the internal loop had been deleted, ion binding to the mutant RNA results in decreased, rather than increased, exposure of the branch site residue to solvent. These data further support the role of the internal loop in defining branch site conformation of the Group II intron. The specific bound Mg^{2+} may play a bivalent role: facilitates the extrahelical conformation of the branch site and has the potential to act as a Lewis acid during splicing.

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1. Introduction

Group II introns are multidomain RNA metallozymes that catalyze their own excision from their primary transcripts by a two step mechanism closely resembling that catalyzed by the spliceosome [1,2]. Activity relies upon the presence of metal ions [3], which are involved in structural and catalytic roles, and formation of specific long range interactions [1,4]. The first splicing step is initiated by the attack of the 2OH of an unpaired adenosine (A880) of the ai5γ Group II intron located in Domain 6 (D6) (Fig. 1).

The results of solution NMR experiments imply structural similarities between the native branch site helix within D6 of the Group II intron [5] and the functionally analogous region in the spliceosome [6,7]. The branch site adenosine of each system is in an extrahelical position, and the two flanking nucleotides participate in intrahelical stacking. The finding that the two branch site regions share common structural features strengthens the argument that they share a common ancestor. In contrast, the solution structure of a D6 construct from which the internal loop had been removed depicted the branch site adenosine stacked within the helix, although with its 2OH exposed; catalytic activity of a Group II intron containing the D6 from which the internal loop was deleted was decreased by ~27% compared with the native D6 sequence [8].

Metal ions play a crucial role in RNA function [2,3,9]. For folded RNA molecules, the pathway for adopting proper tertiary structure, and the stabilization of that structure, depend on specific and nonspecific interactions with certain classes of metal ions [10]. Mg^{2+} is especially suitable for neutralizing the negative charge associated with the RNA backbone because it is the most abundant intracellular multivalent cation and because it has the highest charge density of all biologically available ions. Three general modes of Mg^{2+} binding to RNA have been described: a "diffuse binding" mode providing charge screening to overcome electrostatic repulsion between RNA backbone segments, which is essential for both secondary and tertiary structure formation and can be fulfilled by a diverse range of cations; an "outer sphere site binding" mode involving specific coordination of anionic ligands to hydrated Mg^{2+}, in which Mg^{2+} can often be replaced by cobalt (III) hexammine, an exchange-inert mimic of hydrated Mg^{2+}; and an "inner sphere site binding" mode, characterized by one or more direct contacts between a specific multivalent ion and ligand without intervening water molecules [9,11].

Ion-binding sites in D6 of the full-length ribozyme identified by chemical probing experiments [12] correspond with those in the isolated D6 domain [5,8]. Three regions of D6 have been implicated in site binding of multivalent metal ions: 1) the tetraloop, which forms the η–η′interaction with a region of D2 [5,8,12,13]; 2) the internal loop, which impacts on branch site structure [5,12,14]; and 3) the branch site region, the source of the nucleophilic 2OH [5,8,12]. Although the branch site region from the D6 stem loop from which the internal loop has been deleted is likely to have a different structure than the native context, it includes an ion-binding site [8] that binds Mg^{2+} with a K_d of $\sim 4.2 \times 10^{-3}$ M [15].

Little is known about the specific ion-binding characteristics of the native branch site region of D6 and their role in effecting branch site

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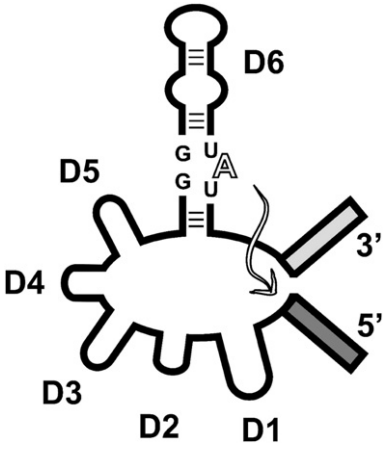

Fig. 1. Scheme of the ai5γ Group II intron. D6 includes the branch site adenosine. The arrow indicates the nucleophilic attack of the 2OH of the branch site adenosine towards the 3′phosphate group of the 5′exon during the first step of splicing.

conformation. Specific RNA-metal ion interaction at this site is likely to play an important role in positioning of the 2OH of A880, in formation of tertiary contacts, and in activation of the 2OH, *i.e.*, for Lewis acid chemistry of the metalloenzyme. Here, we address the specificity of ion binding by the branch site region of the isolated D6 stem loop of the ai5γ Group II intron. Although a small component of a large ribozyme, the stem loop is structurally stable and thus its ion-binding properties are likely to reflect properties in the intact structure. The results presented here demonstrate that the branch site region specifically chelates the alkaline earth metal ions Mg^{2+} and Ca^{2+} by inner sphere coordination and leads to speculation about Lewis acid chemistry of the metal ion in the branching reaction.

2. Experimental details

2.1. Sample preparation

RNA strands containing 2-aminopurine were purchased from Dharmacon and deprotected by the recommended procedure. RNA was dissolved in 10 mM sodium phosphate, 0.1 mM EDTA, pH 6.4. Oligomers (3 μM) were then folded by heating to 90 °C for 3 min and cooling down slowly to 25 °C prior chilling on ice. RNA was dried in vacuum and dissolved in an aqueous NaCl solution (100 mM). The presence of a single fold was verified by nondenaturing PAGE, and the hairpin structure was identified via NOE cross peaks of the tetraloop [16].

RNA for homonuclear NMR samples D6, $D6_{del}$, and $D6_L$ were purchased from Dharmacon. Integrity of the RNA oligomers was tested by denaturing PAGE. RNA pellets were dissolved in high salt buffer (10 mM sodium phosphate, 10 mM EDTA, 1 M sodium chloride, pH 6.4) and then exchanged into NMR buffer (10 mM sodium phosphate, 0.1 mM EDTA, 100 mM sodium chloride, pH 6.4). The RNAs were then folded by heating to 90 °C for 3 min and cooling down slowly to 37 °C before chilling on ice. After folding, the samples were suspended in 90% H_2O/10% D_2O (v/v). Final concentration of the RNA in the NMR samples was ≈0.85 mM. Microvolume NMR tubes (Shigemi) were used for data collection. The presence of a single fold of all RNA samples was verified by nondenaturing PAGE, and the hairpin structure was identified via NOE cross peaks of the tetraloop [16].

2.2. Fluorescence spectroscopy

A Cary Eclipse (Varian) fluorescence spectrometer was used for the 2-aminopurine experiments. The quartz cuvettes (Starna Cells, 4 mm square) contained 130 μl buffered RNA solution. Excitation occurred at 306 nm and 25 °C. Emission was detected at 367 nm. All experiments were performed at least three times and averaged. Metal ion titrations were performed as indicated. Metal ion concentration ranged from 0.01 mM–5 mM.

2.3. NMR spectroscopy

All NMR data were acquired on a 600 MHz Varian Unity Plus spectrometer (National High Magnetic Field Laboratory, Tallahassee, Florida). Exchangeable protons were observed at 4 °C with samples in aqueous buffer including 10% D_2O to obtain lock. A jump-return echo pulse sequence [17] was used for water suppression. NMR data were processed using Varian VNMR and Spinworks software.

3. Results and discussion

3.1. Metal ion specificity at the branch site

In previous studies, we monitored fluorescence of 2-aminopurine (2ap), an analogue of adenine, the fluorescence of which is quenched by stacking of bases and enhanced by increased solvent accessibility, as a probe for branch site position [5]. NMR results showed that substitution of 2ap for the branch site base did not perturb the overall structure of D6. Through a combination of 2ap fluorescence and NMR experiments, we determined that the branch site A flanked by two GU base pairs from ai5γ Group II intron is in an extrahelical conformation, and that the surrounding bases are all stacked intrahelically. The extrahelical conformation of the branch site residue is enhanced by the presence of metal ions. Moreover, this particular motif depends upon the presence of the internal loop three base pairs away: when the internal loop is deleted, 2ap fluoresced ~30% less than full-length $D6_{2ap}$, suggesting that the branch site adenosine in the D6 stem loop from which the internal loop had been omitted is less solvent exposed than in the full-length RNA [5]. The NMR structure of the truncated stem loop has verified intrahelical stacking of the branch site residue, flanked by G·U pairs [8]. Several cross peaks in spectra of exchangeable protons of the two constructs have similar chemical shifts but have been but are attributed to different interactions [5,8]; however, there are clear structural differences between the branch site regions of the two constructs, and different assignments for analogous residues most likely reflect the different chemical environments of individual residues.

In order to characterize the ion-binding behavior of the branch site region in the native domain sequence, we have focused on the branch site RNA interaction with different metal ions. Mg^{2+} ions facilitate both steps of splicing by the Group II intron [3], and Ca^{2+} selectively supports the first transesterification step [18]. For this reason, we titrated Ca^{2+} into a 3 μM 2ap RNA solution and observed a similar effect as had been seen for Mg^{2+}, specifically, the fluorescence of $D6_{2ap}$ increased by ~10% upon addition of 5 mM Ca^{2+} (Fig. 2). Therefore, in spite of the difference in ionic radius (Table 1), the two ions elicit the same response, suggesting that they fulfil the same structural role at this site.

Addition of up to 5 mM $[Co(NH_3)_6]^{3+}$, Mn^{2+}, Zn^{2+}, and Tb^{3+} resulted in no measurable increase in fluorescence. This result suggests that $[Co(NH_3)_6]^{3+}$, Mn^{2+}, Zn^{2+}, and Tb^3 do not bind to the branch site region. The finding is not expected because the major groove of the GU base pairs is the most common metal ion-binding motif [19]. For example, the upstream site of cleavage of all group I introns is characterized by a conserved GU. It has been shown that Mn^{2+} and Mg^{2+} bind specifically at the wobble base pair [20]. Tandem GU base pairs in P5b of the same intron indicated complexation of the major groove with $[Co(NH_3)_6]^{3+}$ [21]. $Co(NH_3)_6^{3+}$ ion was localized at the branch site in the crystal structure of a highly modified construct investigated by Zhang and Doudna [22], suggesting that the RNA-metal ion interaction it mimicked did not involve inner sphere binding — *i.e.* a different mode of ion binding from what we observe in the native stem loop. Non-linear Poisson–Boltzmann calculations of electrostatic profiles of

Table 1
Metal ions used in 2-aminopurine fluorescence experiments

M^{n+}	M^{n+}radius in Å	Coordination	Exchangeable ligands	Properties	References
Mg^{2+}	0.65	6	+	[a]	[3]
Ca^{2+}	0.99	6	+	[b]	[18]
$[Co(NH_3)_6]^{3+}$	2.77	6	–	[c]	[33,34]
Mn^{2+}	0.8	6	+	[d]	[35,36]
Zn^{2+}	0.74	4, 6	+	[e]	[37,38]
Tb^{3+}	0.92	≤9	+	[f]	[39–41]

[a] Facilitates both steps of splicing.
[b] Supports selectively the first step of splicing.
[c] Mimics fully hydrated Mg^{2+}.
[d] "Soft" ion which prefers typically "soft" ligands (nitrogen over oxygen). $[Mn(H_2O)_6]^{2+}$ has been shown to replace $[Co(NH_3)_6]^{3+}$ and $[Mg(H_2O)_6]^{2+}$.
[e] Like Mg^{2+}, Zn^{2+} does not show biological relevant redox activity.
[f] In some RNAs lanthanide (Ln^{3+}) binding sites overlap with Mg^{2+} binding sites.

GU wobble pairs in helices revealed the importance of the surrounding sequence on the overall electronegativity [23]. In our experiments neither the cobalt complex nor Mn^{2+}, Zn^{2+}, Tb^{3+} induced a measurable changed in 2ap fluorescence of $D6_{2ap}$. This strengthens the argument that in solution the branch site of D6 binds partially dehydrated Mg^{2+}, which, in turn, impacts on its conformation.

As a control, we titrated Mg^{2+}, Ca^{2+}, Mn^{2+}, $[Co(NH_3)_6]^{3+}$, Zn^{2+}, and Tb^{3+} into a mutated D6 stem loop which had a fully base paired stem, *i.e.* the internal loop was deleted and the branch site 2ap forms a complementary base pair with an added U in the 5′side of the stem. In this context, 2ap exhibited no change in fluorescence upon addition of any of these metal ions up to 5 mM. Taken together, the results of our metal ion titrations lead us to conclude that conformational change in the branch site region resulting in extrusion of the adenosine residue is dependent upon specific interaction with, and partial dehydration of, alkaline earth metal ions.

The internal loop assists in, but appears not to be essential for, ai5γ catalysis [8], but its function is unknown. Extrahelical branch site conformation depends upon the presence of the internal loop [5]; what is not known is whether it also forms any tertiary interactions with other structural elements within the intron.

3.2. The internal loop generates a specific local environment for the branch site region

Results of phosphorothioate interference studies of the ai5γ Group II intron by Chanfreau and Jacquier had suggested that one phosphate oxygen atom of the internal loop of D6 was more likely to play a structural than a catalytic role during the first step of splicing. DMS modification studies of the PI.LSU/2 Group II intron suggest tertiary interaction of the internal loop of D6 [24]. It is striking that the PI.LSU/2 and ai5γ branch site and internal loop sequences comply. Although the sequence of the internal loop of D6 of ai5γ is not conserved among Group II introns [25,26], it is possible that whatever role it performs in splicing might be adopted by analogous elements in other Group II sequences.

In contrast with the increase in fluorescence of $D6_{2ap}$ upon addition of Mg^{2+} or Ca^{2+}, addition of 5 mM Mg^{2+} or Ca^{2+} to $D6_{2ap,del}$ resulted in a ~8% decrease in fluorescence [5,8]. As was the case with $D6_{2ap}$, addition of Mn^{2+}, $[Co(NH_3)_6]^{3+}$, Zn^{2+}, or Tb^{3+} up to 5 mM resulted in no change of the fluorescence signal in $D6_{2ap,del}$ (Fig. 2). From these data, we conclude that the preference remains for the alkaline earth ions Mg^{2+} and Ca^{2+}, which are likely to be partially dehydrated upon specific interaction with the nucleotides in the branch site region.

3.3. Impact of Mg^{2+} on full-length D6 measured by NMR

We next monitored the changes of the imino proton resonances in three D6 constructs to monitor the chemical micro-environment upon addition of Mg^{2+} (Fig. 3a): the native D6 (D6), D6 without internal loop ($D6_{del}$), and D6 from which the branch site nucleotide was deleted, forming a complementary stem ($D6_L$). 1D ^{1}H NMR spectra of exchangeable protons of D6 indicated no major spectral change, suggesting no global structural perturbation upon addition of Mg^{2+} and Ca^{2+} up to 5 mM to any of the stem loops studied. However, the resonances of a number of imino protons attributed to bases within the internal loop and branch site region shifted upon addition of these metal ions.

Changes in the chemical shifts of imino protons in native D6 upon addition of Mg^{2+} were described previously [5]. Fig. 3b summarizes the relative $\Delta\delta$ changes of imino proton resonances of $D6_L$ (left), D6 (middle), and $D6_{del}$ (right) upon addition of Mg^{2+}. Chemical shifts of imino protons in $D6_L$ are in strong agreement to those found in the full-length D6. Only the shift of G858, which immediately precedes the internal loop in the native D6, differs in sign. This indicates that upon Mg^{2+} addition, the chemical local environment of the detected imino protons within the internal loop and the tetraloop is similar in D6 and $D6_L$. In contrast with the downfield shifts of U879, G856, and G857 of D6 upon addition of Mg^{2+}, these same imino protons exhibited upfield chemical shift changes in $D6_{del}$ upon addition of Mg^{2+}. In D6 and $D6_{del}$ the imino protons of G855 and U881 shift both upfield. However, the G855h1 shift in D6 is significantly stronger.

These findings are consistent with differences in the imino proton micro-environments of the branch site region of D6 and $D6_{del}$ upon addition of Mg^{2+}: imino protons of D6 are more deshielded upon complexation with Mg^{2+}, whereas those from $D6_{del}$ become more shielded. An upfield shift of imino protons, particularly of G·U and other noncanonical pairs, is commonly seen upon binding of Mg^{2+} [27–29]. However, the downfield shifts observed for imino protons of the D6 branch site in the presence of the internal loop suggest the possibility of overall difference in geometry, such as a difference in the degree of helical twist. The results support our earlier findings that the structural distinctions in D6 and $D6_{del}$ rely on the presence of the internal loop in D6 [5]. In addition, results of Sigel and coworkers underscore the structural differences between native D6 and a D6 construct from which the internal loop was omitted [8]. Mg^{2+} titration experiments confirmed metal ion binding within the branch site

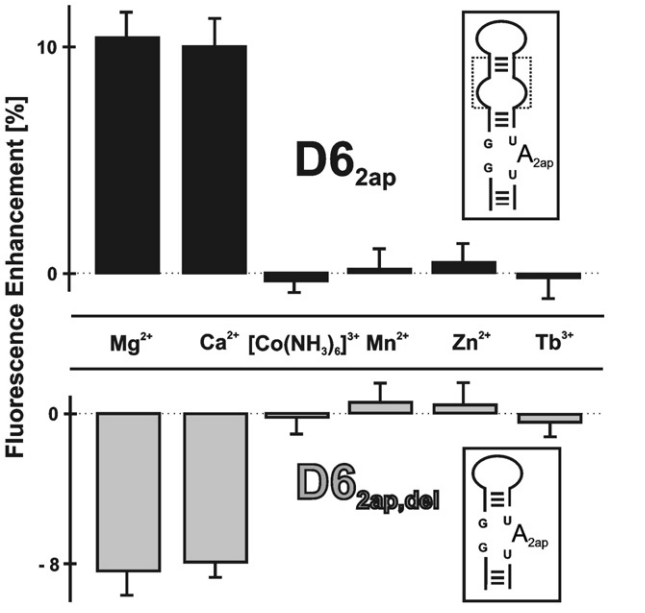

Fig. 2. Relative change of the 2ap fluorescence of the native and mutated $D6_{2ap}$ ($D6_{2ap}$ — top, $D6_{2ap,del}$ — bottom) upon metal ion addition. Excitation occurred at 306 nm. The 2ap emission was monitored at 367 nm. Each experiment was performed at least three times. The insets contain the schemes of the corresponding RNA constructs; the dotted box indicates the region of the sequence that was deleted in $D6_{2ap,del}$.

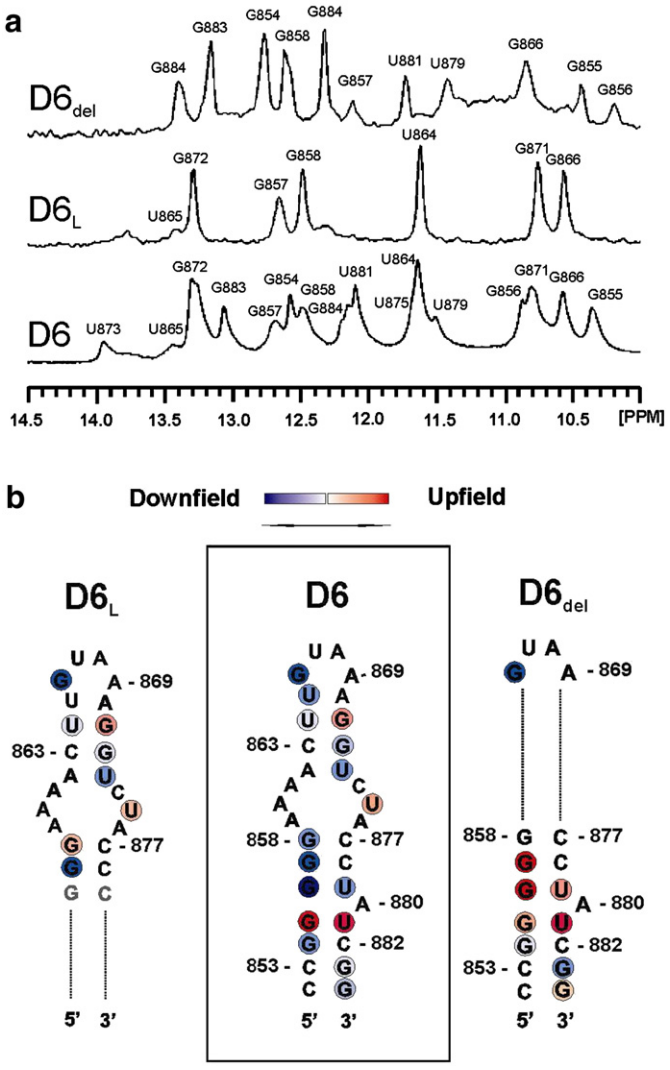

Fig. 3. ^{1}H NMR spectroscopic investigation of D6. a) Imino proton spectra of $D6_{del}$ (top), $D6_L$ (middle), and D6 (bottom) acquired at 4 °C and 0 mM Mg^{2+}. b) Mapping of the change in chemical shift of the imino protons upon addition of 5 mM Mg^{2+}. The dotted lines denote nucleotides deleted from the native stem loop sequence. Red indicates nucleotides associated with upfield-shifted resonances, and blue corresponds to a downfield shift. The pattern of chemical shift changes in the branch site region is very different in the D6 fragment from which the internal loop has been deleted.

region but no evidence for the mode and specificity of metal ion binding at the branch site has been reported [8].

3.4. The branch site region specifically binds a functional magnesium ion

Similarities among chemical mechanisms and certain structural features shared by the spliceosomal branch site duplex [7], the Group II intron branch site [5], and the active site of the Group I intron [30] in solution fuel the speculation about their common ancestry. Crystallographic studies by the Cech laboratory revealed that the active site of the *Tetrahymena* Group I intron binds a specific metal ion which appears to be coordinated to three separate phosphate groups, brought together by the RNA tertiary structure [31]. In addition, this Mg^{2+} ion binds the ribozyme even in the absence of substrate.

Questions remain about the exact transesterification mechanism for each splicing step in the Group II intron [25]. However, the chemical reaction for the first step includes an in-line S_n2 nucleophilic attack of the 2OH of A880 on the 5′splice site, accompanied by an inversion of the configuration at the phosphate. 3′-sulfur substitutions of the 5′ splice sites confirmed that Group II intron ribozymes are metalloenzymes [2]. By analogy with a two-metal-ion phosphoryl transferase mechanism of the DNA polymerase I 3′,5′-exonuclease domain complexed with single-stranded DNA, derived from a crystal structure, in which the Mg^{2+} ions are positioned for catalysis in an inner sphere complex near the scissile phosphate [32], Steitz and Steitz (1993) hypothesized that Group II intron catalysis is facilitated by two divalent metal ions. The first Mg^{2+} ion is proposed to activate the 2OH of A880, whereas the second Mg^{2+} ion stabilizes the leaving oxyanion and pentacovalent intermediate. Thus, based upon these observations, it appears that the specificity of this site for Mg^{2+}, as opposed to multivalent ions with other properties, is of chemical, as well as structural, importance.

Alternatively, results of sulfur substitution experiments led to the conclusion that only one Mg^{2+} ion is involved in catalysis of the first splicing step [2]. By either model, it is clear that Mg^{2+} ion acts as a Lewis acid and must be positioned in the active site. Our findings are consistent with the requirements for a potential metal ion-binding site that could position a catalytically active Mg^{2+} ion for structural and/or chemical purposes.

Evidence from these studies suggests that the Group II intron branch site region chelates Mg^{2+} by inner sphere site binding, which induces enhanced exposure of the branch site A880. Thus the specific metal ion may help position the 2′-hydroxyl group of the branch site adenosine and could adopt the role of a Lewis acid, thereby facilitating the first step of splicing. Consequently, Mg^{2+} and the branch site region benefit from one another — a molecular symbiosis.

4. Conclusions

Our results indicate that the Group II intron branch site complexes Mg^{2+} and Ca^{2+} specifically.

Binding specificity does not rely on the presence of the internal loop of D6. However, the presence of internal loop determines the degree of extrahelicity of the branch site adenosine upon Mg^{2+} addition. Moreover, it is likely that metal ion binding occurs by inner sphere mode. These findings support the importance of Mg^{2+} in the native branch site structure and allow us to speculate that the inner sphere bound metal ion adopts a catalytic role as a Lewis acid during splicing.

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