Outersphere and innersphere coordinated metal ions in an aminoacyl-tRNA synthetase ribozyme

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ABSTRACT

Metal ions are essential cofactors for various ribozymes. Here we dissect the roles of metal ions in an aminoacyl-tRNA synthetase-like ribozyme (ARS ribozyme), which was evolved in vitro. This ribozyme can charge phenylalanine on tRNA in cis, where it is covalently attached to the 5'-end of tRNA (i.e. a form of precursor tRNA), as well as in trans, where it can act as a catalyst. The presence of magnesium ion is essential for this ribozyme to exhibit full catalytic activity. Metal-dependent kinetics, as well as structural mappings using Tb3+ in competition with Mg2+ or Co(NH3)63+, identified two potential metal-binding sites which are embedded near the tRNA-binding site. The high affinity metal-binding site can be filled with either Mg2+ or Co(NH3)63+ and thus the activity relies on a metal ion that is fully coordinated with water or ammonium ions. This site also overlaps with the amino acid-binding site, suggesting that the metal ion plays a role in constituting the catalytic core. The weak metal-binding site is occupied only by a metal ion(s) that can form innersphere contacts with ligands in the ribozyme and, hence, Mg2+ can enhance ribozyme activity, but Co(NH3)63+ cannot. The experiments described in this work establish the roles of metal ions that have distinct coordination properties in the ARS ribozyme.

INTRODUCTION

Although a number of experiments have been reported to date to explore the general roles of metal ions in ribozymes (1–4), it is still unclear in some cases whether metal ions contribute to structural or chemical roles in each ribozyme-catalyzed reaction. In the case of the hammerhead ribozyme, for instance, it had originally been thought that divalent metal ions exclusively play catalytic roles, i.e. they directly participate in the reaction (5–7). However, recent studies have shown that high concentrations of monovalent ions can support catalysis, suggesting that structural roles of the divalent metal ions cannot be ruled out (8–10). Metal ions in the hairpin ribozyme appear to play structural roles rather than catalytic roles, as suggested by biochemical and X-ray structural studies (8,11–14). In order to shed light on the diverse roles of metal ions in ribozymes, it is of interest to investigate not only naturally occurring, but also artificially evolved ribozymes.

We have evolved in vitro an artificial precursor tRNA (pre-tRNA) that exhibits self-phenylalanlation (Fig. 1) (15). This isolated pre-tRNA consists of a catalytic 5'-leader sequence and tRNA domain. The in vitro evolution yielded a single family of ribozymes, of which pre-24, a representative clone, was further characterized. Although the original tRNA domain was designed to be a full-length tRNA (referred to as otRNA), the tRNA sequence domain in pre-24 was truncated during the evolution (we refer to this tRNA as rtRNA; Fig. 1, right). We found that the 5'-leader sequence of pre-24, referred to as r24 (Fig. 1, left), is able to aminoacylate otRNA, as well as rtRNA, in trans. It can discriminate between the phenylalanine substrate and other natural amino acids that have distinct side chains, thus acting as a PheRS ribozyme. Moreover, biochemical analyses of pre-24 (as well as r24) revealed the essential catalytic core that recognizes both the amino acid and tRNA substrates (Fig. 1) (16). In light of the essential roles of metal ions in RNA chemistry, we wondered whether Mg2+, which was originally used for the in vitro evolution, is critical to the ribozyme-catalyzed aminoacylation activity. Herein we have made an effort to gain insights into the role of metal ions in this ribozyme.

MATERIALS AND METHODS

Materials

Metal chlorides used in this study were purchased from Sigma and were of the highest purity available. Enzymes were purchased from Promega or prepared in house. Other chemicals were purchased from Sigma and pre-mixed acrylamide solutions (Seque-gel) were purchased from National Diagnostics. Synthesis of the amino acid substrate has been reported elsewhere (15).
RNA preparation

All RNAs were transcribed from PCR-amplified templates by T7 RNA polymerase either in the absence or presence of [α-32P]UTP. After RNase-free DNase treatment for 15 min, transcripts were purified by 6–10% denaturing PAGE. For 5'-labeled RNA preparation, RNAs were treated with calf intestinal alkaline phosphatase, then phosphorylated using T4 polynucleotide kinase in the presence of [γ-32P]ATP and purified by 6–10% denaturing PAGE. For the construction of r24 that lacks U85–U90 (r24p), the r24 ribozyme acts as a trans-catalyst for the aminocaylation of tRNA. The r24 ribozyme consists of three stem–loops (P1–P5 and L1–L3) connected by two junction regions (J12a and J2a/3). The base pairing between G37–C75 at the 5'-terminus of the tRNA (the base numbers of the tRNA domain are denoted in subscript) and G70–U72 (green rectangle) in L3 is essential for catalysis, and this interaction is indicated by a green arrow. U59–U62 and U67–U68 in J2a/3 and the L3 loop, respectively, are responsible for recognition of the phenylalanine substrate (highlighted in red circles). The original structure of an artificial orthogonal suppressor tRNA (otRNA) is shown. Bold and italic bold bases in the tRNA domain were deleted and mutated (A53C and G54A), respectively, during in vitro evolution, and this truncated tRNA is referred to as rtRNA. The v1-tRNA is derived from rtRNA, in which the original anticodon region of otRNA (U33–G43) was restored, but the remaining point mutations and deletions in rtRNA were maintained (15). The base numbers of otRNA are assigned according to the tRNA numbering rule (33). AC, anticodon loop; V, variable region; T, TPC loop.

General kinetic assays

General kinetic assays were carried out under single turnover conditions by using 0.5–1 μM self-aminocaylating ribozyme and 5 mM N-biotinylated L-phenylalanyl cyanomethyl ester (biotin-L-Phe-CME) as their final concentrations. Under this condition and in the presence of 100 mM Mg2+ the observed rate constant (k_{obs} = 0.07 min^{-1}) represents a value close to k_{cat} = 0.13 ± 0.014 min^{-1} (k_{cat} = 2.8 ± 0.61 mM). Note that above 5 mM biotin-L-Phe-CME the substrate tends to precipitate in the solution (generally 5–20 mM), was mixed with 7 μl of 4× EK buffer (200 mM EPPS, 50 mM KCl, pH 7.5) and an appropriate volume of DEPC-treated water to give a final volume of 19.6 μl. This RNA solution was heated at 95°C for 5 min and then cooled to 25°C over 5 min. Various concentrations of 5× MgCl2 buffer (5.6 μl) were then added and the mixture was equilibrated at 25°C for 5 min. Reactions were initiated by the addition of 2.8 μl of 50 mM biotin-L-Phe-CME in ethanol/water (1:1) to the above RNA/metal solution (the total volume was 28 μl and the final concentration of ethanol was 5%). Aliquots of 5 μl were removed at various time points, quenched with 5 μl of PUE buffer (25 mM PIPES, 8 M urea, 50 mM EDTA) and ethanol precipitated twice. The pellets were then dissolved in 5 μl of MEUS buffer (25 mM MOPS, 8 M urea, 5 mM EDTA, 10 μM streptavidin, pH 6.5). The resulting products were resolved by 6 or 10% denaturing PAGE at 4°C, where the gel surface temperature was kept below 20°C. Note that under this electrophoresis condition the biotin–streptavidin interaction was not disturbed, but the RNA structures were denatured and, hence, the aminoacylated RNA can be clearly separated from unreacted RNA. The bands were quantified using a Molecular Imager FX (Bio-Rad). Rate constants were determined by taking five data points from the linear regions of the time course using Kaleida Graph (Abelbeck Software). Since the cis-acting (self-modifying) ribozyme is intrinsically limited to a single turnover reaction, and since its active site is saturated with the tRNA substrate when it is folded, the slope of the linear plot for the fraction of the aminoacylated cis-ribozyme versus time corresponds to the rate constant (k_{obs}).

For Co(NH3)63+-dependent kinetics, MgCl2 was replaced by Co(NH3)63+, and 10 mM EDTA was added to the solution in order to remove contaminant metals. Trans reactions were carried out as follows: 2 μM r24Δ6 or its mutants (Fig. 5) and 0.5 μM v1-tRNA were independently denatured at 95°C for 5 min, then cooled to 25°C for 5 min. Then these RNAs were independently folded by adding 100 mM MgCl2 at 25°C for 5 min. After mixing the ribozyme with tRNA solution, the reactions were initiated by adding 5 mM biotin-L-Phe-CME. The remaining procedure was the same as described above. In the trans reactions, we also used higher and lower concentrations of the trans-ribozyme (1–4 μM); however, the observed rate constants were the same as those of 2 μM ribozyme and no multiple turnover was observed (presumably dissociation of the ribozyme from tRNA does not occur). Although we still need more extensive kinetic studies in order to gain insights into the nature of this trans system, the above result indicates that the trans reaction catalyzed by this ribozyme construct is apparently limited to the single-turnover reaction. Therefore, the kinetic behavior of the trans system is the same as the cis system, except that the preparation of mutants in the former system was easier than in the latter system. An additional note is that the background rate was negligible under the conditions described above. After 3 h incubation of otRNA (or any other tRNA) with 5 mM biotin-L-Phe-CME, an approximately 0.01% yield (which is our minimum detection limit) of biotin-L-Phe-otRNA was observed, giving an estimated value for k_{un} of 5.5 × 10^{-7} min^{-1} (15).

Tb3+- and Mg2+-mediated RNA cleavage

The 5'-labeled pre-24 or r24Δ6 was dissolved in EK buffer, denatured at 95°C for 5 min, then cooled to 25°C over 5 min. For Tb3+-mediated cleavage, RNA was allowed to fold in the
presence of 50 or 100 mM MgCl₂ at 25°C for 5 min. Cleavage reactions were initiated by adding freshly prepared TbCl₃ solution, and incubated at 25°C for 1 h. For Mg²⁺-mediated cleavage, the pH of EK buffer was elevated to 9, with incubation at 25°C for 4 h. Reactions were stopped by addition of 100 mM EDTA, followed by ethanol precipitation. Cleavage products were resolved by 12% denaturing PAGE for 3.5 h, and analyzed using a Molecular Imager FX (Bio-Rad). For Tb³⁺–Mg²⁺ and Tb³⁺–Co(NH₃)₆³⁺ competition assays, experiments were carried out in a manner similar to the above experiment with the following modifications. RNA in EK buffer was equilibrated at various concentrations of Mg²⁺ (0–50 mM) or Co(NH₃)₆³⁺ (0–25 mM) at 25°C for 5 min. The reaction was initiated by adding 50 or 100 µM TbCl₃, then incubated at 25°C for 1 h. Cleavage profiles for both concentrations of Tb³⁺ were identical.

Assay for dependence of self-aminoacylation on monovalent ions

Stock solutions of NaCl (5 M), LiCl (5 M) and KCl (3.8 M) were prepared prior to the reaction. For the aminoacylation assay, reactions were set up as described in the general kinetic assays, except that the volume of the RNA solution was adjusted accordingly in order to reach high concentrations of the monovalent ion, and 10 mM EDTA was added to remove multivalent metal ion contaminants.

RESULTS

Metal species dependence
We first investigated metal species dependence of pre-24. We chose seven distinct divalent metal ions, Mg²⁺, Ca²⁺, Mn²⁺, Co²⁺, Cu²⁺, Zn²⁺ and Cd²⁺. Both alkaline earth metal ions are effective (Fig. 2A, lanes 1 and 2), with Mg²⁺ being 2-fold more active than Ca²⁺. Mn²⁺ can also support catalysis (lane 3), but the observed activity is considerably less than those observed for the alkaline earth metal ions. Neither the presence of other transition metal ions nor the absence of Mg²⁺ supported catalysis (lanes 4–8). This preliminary study seems to indicate that alkaline earth metal ions assisted aminoacylation activity better than transition metal ions under the conditions that we chose to test.

Co(NH₃)₆³⁺ mimics the hexahydrated form of Mg²⁺, Mg(H₂O)₆²⁺. We have previously shown that in an acyltransferase ribozyme (ATRib) Mg²⁺ can be substituted with Co(NH₃)₆³⁺, exhibiting a half maximal activity at 0.35 mM Co(NH₃)₆³⁺ compared with 14 mM Mg²⁺ (17). It has been also demonstrated that the catalytic activity of the hairpin ribozyme is supported by Co(NH₃)₆³⁺ (12,18,19). Activity observed using Co(NH₃)₆³⁺ in both ribozymes indicates that the metal ion(s) playing a critical role(s) in catalysis binds through an outersphere coordination site(s). On the other hand, in some ribozymes, such as hammerhead and GlnRS-like ribozyme (which is an artificially evolved ribozyme), Co(NH₃)₆³⁺ can act as a strong inhibitor due to its inability to exchange the ammonium ligands, i.e. these ribozymes require innersphere coordination to the metal-binding sites (20–22). Therefore, Co(NH₃)₆³⁺ is a good probe to investigate the coordination of Mg²⁺ in ribozyme catalysis. We thus tested whether pre-24 can function in the presence of Co(NH₃)₆³⁺ in the range 1–100 mM, and the activity was compared with the Mg²⁺-dependent reaction (Fig. 2B). Interestingly, the observed activity at 1 mM Co(NH₃)₆³⁺ was slightly higher than the activity at 1 mM Mg²⁺ (lane 1 versus lane 6), but an increase in the Co(NH₃)₆³⁺ concentration does not enhance the activity

Figure 2. Metal ion-dependent activity of pre-24. (A) Divalent metal ion dependence of pre-24. Self-aminoacylation was carried out in the presence of 100 mM metal (lanes 1–7) or no metal (lane 8). a, aminoacylated pre-24 complexed with streptavidin (SAv); b, unreacted pre-24; N.D., not detectable. Reactions were carried out in the presence of 1 mM pre-24, incubated with 5 mM biotin-Phe-CME at 25°C for 1 h. (B) Mg²⁺ or Co(NH₃)₆³⁺-dependent self-aminoacylation activity of pre-24. Lanes represent samples incubated with Mg²⁺ (lanes 1–4) or Co(NH₃)₆³⁺ (lanes 6–9) at the following concentrations: 1, 10, 20 and 50 mM. Samples in lanes 5 and 10 were incubated with 50 mM Mg²⁺ and Co(NH₃)₆³⁺ in the presence of 100 mM EDTA, respectively. (C) Catalytic activity of pre-24 as a function of Mg²⁺ (filled circle, red) or Co(NH₃)₆³⁺ (open triangle, black) concentration. Data were fitted to smooth interpolations. The inset shows activity below 120 mM Co(NH₃)₆³⁺ and 7 mM Mg²⁺. (D) Hill analysis. Data were taken from k₉obs observed for 0.5–6.25 mM Mg²⁺ in (C) and the fraction of activity (f) was determined based on the k₉obs (= 0.048 min⁻¹) observed for 50 mM Mg²⁺.
significantly (lanes 6–9). This is in contrast to the observation that an increase in the Mg\(^2+\) concentration results in a gradual increase in the activity (lanes 1–4). Addition of 100 mM EDTA to 50 mM Mg\(^{2+}\), which removes essential Mg\(^{2+}\) from the ribozyme, eliminates the activity completely (lane 5). On the other hand, the same treatment to 50 mM Co(NH\(_3\))\(_6^{3+}\) slightly increases the activity (lane 9 versus lane 10), which is probably due to the removal of contaminants in Co(NH\(_3\))\(_6^{3+}\) (such as multivalent metal ions that act as inhibitors) by EDTA. Most importantly, the addition of EDTA to Co(NH\(_3\))\(_6^{3+}\) does not inhibit the activity, which is consistent with the view that Co(NH\(_3\))\(_6^{3+}\) is the active species for this ribozyme.

The observed difference in the activity between Mg\(^{2+}\) and Co(NH\(_3\))\(_6^{3+}\) led us to further investigate the dependence of the reaction rate upon their concentrations. Thus, the rate constants were determined over a wider range of concentrations of these two metal ions (Fig. 2C). The titration plot ([Mg\(^{2+}\)] versus \(k_{\text{obs}}\)) displayed a biphasic behavior, where the activity rapidly increases up to 20 mM Mg\(^{2+}\), then slowly increases beyond 20 mM. The addition of spermidine did not change the biphasic behavior (data not shown), indicating that non-specific structural Mg\(^{2+}\) does not contribute to this behavior. It should be noted that Hill analysis of the activity below 20 mM (based on five data points) gives a coefficient of 0.97 (Fig. 2D), suggesting that a single metal ion is most likely responsible for the activity in this region. We thus propose that at least two Mg\(^{2+}\) ions are involved in catalysis, where one can bind to a high affinity metal-binding site and the other(s) can bind to a low affinity site(s). In the case where Co(NH\(_3\))\(_6^{3+}\) and EDTA are present, the initial rate is similar to the Mg\(^{2+}\)-dependent profile below 2.5 mM. On the other hand, the activity nearly plateaus at higher concentrations (20–120 mM), although the solubility limit of Co(NH\(_3\))\(_6^{3+}\) made it difficult to determine the activity above 120 mM. Thus, even though Co(NH\(_3\))\(_6^{3+}\) supports catalysis of pre-24, it does not fulfill the same role as Mg\(^{2+}\). Most likely, Co(NH\(_3\))\(_6^{3+}\) can replace the role of the high affinity Mg\(^{2+}\), but it cannot replace the role of the low affinity Mg\(^{2+}\) ion(s). We therefore hypothesize that the high affinity metal-binding site is filled with Mg(H\(_2\)O)\(_6^{2+}\) or Co(NH\(_3\))\(_6^{3+}\), and the other low affinity metal-binding site(s) is occupied by Mg\(^{2+}\) whose water ligands can be exchanged with ligands in the metal-binding site of the ribozyme. To facilitate the discussion below, we refer to the former and latter metal ions as outersphere and innersphere coordinated metal ions, respectively.

**Tb\(^{3+}\) and Mg\(^{2+}\) concentration-dependent cleavage**

Hexacoordinate terbium ion (Tb\(^{3+}\)) has an ionic radius (0.92 Å) that is close to Mg\(^{2+}\) (0.72 Å), and it has the same preference for coordination to oxygen ligands (1,23,24). It has been found in some RNAs that Mg\(^{2+}\)-binding sites fairly overlap with Tb\(^{3+}\)-binding sites. Despite these similarities, Tb\(^{3+}\) is capable of cleaving RNA at neutral pH whereas Mg\(^{2+}\) generally does not. This activity is attributed to a property of Tb\(^{3+}\) that generates the 2'-alkoxide ion in the binding sites, followed by an in-line attack on the 3'-bridging phosphate resulting in RNA cleavage. Thus, Tb\(^{3+}\) cleavage sites can approximate the number of Mg\(^{2+}\)-binding sites, and hence Tb\(^{3+}\)-dependent hydrolytic mapping has recently been used to assess Mg\(^{2+}\)-binding sites (22,25–28). It should be noted, however, that Tb\(^{3+}\)-dependent RNA cleavage reflects two aspects of the RNA structure depending upon the following conditions. In the presence of an excess amount of Mg\(^{2+}\), high concentrations of Tb\(^{3+}\) mainly cleave single-stranded or structurally relaxed regions of folded RNA in addition to Tb\(^{3+}\) localization sites. However, in the presence of a limited amount or absence of Mg\(^{2+}\), low concentrations of Tb\(^{3+}\) cleave more specifically at high affinity metal ion-binding sites, and thus it potentially probes Mg\(^{2+}\)-binding sites. Therefore, the conditions for this series of experiments must be carefully determined in order to assess Mg\(^{2+}\)-binding sites by Tb\(^{3+}\) cleavage mapping experiments.

Based on the above consideration, we first mapped pre-24 with Tb\(^{3+}\) at varying concentrations in the presence of 100 mM Mg\(^{2+}\), where Mg\(^{2+}\) most likely saturates the high affinity metal-binding site (Fig. 3A). High concentrations of Tb\(^{3+}\) (0.5–5 mM) in the presence of 100 mM Mg\(^{2+}\) cleaved single-stranded regions in the proposed secondary structure, as expected (Fig. 3A, lanes 1–3 and see also Fig. 1, left). We also identified the V- and D-loop regions of the tRNA domain that were accessible by Tb\(^{3+}\) (Fig. 3A, lanes 1–3). This is consistent with our previous report that the secondary structure of the tRNA domain maintains a cloverleaf-like secondary structure (16). At low concentrations of Tb\(^{3+}\) (0.1 and 0.04 mM) the cleavage intensity in most regions decreased (lanes 4 and 5), suggesting that these regions are potential Mg\(^{2+}\)-binding sites. In contrast, cleavages at A\(_{14}\) and A\(_{15}\) in the D-loop region remained the same at various concentrations of Tb\(^{3+}\) (lanes with subscript numbers indicate tRNA bases). Although it has been observed that Tb\(^{3+}\) and Pb\(^{2+}\) often cleave in the D-loop region of tRNA, the lack of competition with Mg\(^{2+}\) observed in this study suggests that the Tb\(^{3+}\) cleavage sites found in the D-loop region in the pre-24 tRNA domain are unlikely high affinity Mg\(^{2+}\)-binding sites (in spite of their extraordinarily high affinity for Tb\(^{3+}\)). It is important to note that the presence of Tb\(^{3+}\) at a high concentration of 5 mM does not perturb the structure of pre-24 significantly (lane 1), and a low concentration as low as 40 μM Tb\(^{3+}\) (lane 5) is sufficient to induce specific RNA cleavage.

The above Tb\(^{3+}\)-concentration-dependent cleavage experiment suggests that the potential Mg\(^{2+}\)-binding sites of the 5'-leader domain are in the C89 linker region (A86–U90), L3 region (U67–C74) and J2a/3 region (A60–U62). To verify the overlaps of both Mg\(^{2+}\) and Tb\(^{3+}\) localization sites, we employed Mg\(^{2+}\)-dependent RNA cleavage under basic conditions (Fig. 3B). Although Mg\(^{2+}\) does not cleave pre-24 at neutral pH, elevating the pH to 9 effectively induced cleavage in the presence of high Mg\(^{2+}\) concentrations. Notably, the cleavage profile observed for Mg\(^{2+}\)-dependent cleavage is nearly identical to that observed for Tb\(^{3+}\) (Fig. 3A versus B). This confirms that Tb\(^{3+}\) and Mg\(^{2+}\) localize at the same sites, which in turn agrees with the idea that Tb\(^{3+}\) can be used effectively to map the Mg\(^{2+}\)-binding sites at neutral pH.

Our previous studies on the structure–function relationship of pre-24 have revealed that the L3 and J2a/3 regions are responsible for the recognition of both the tRNA and Phe substrates. Therefore, the Tb\(^{3+}\) and Mg\(^{2+}\)-dependent cleavages observed within these regions may represent critical Mg\(^{2+}\) localization sites in the ribozyme. On the other hand, both Tb\(^{3+}\) and Mg\(^{2+}\) intensively cleaved C89 in the linker...
region between the ribozyme and tRNA domains. We have previously shown that the r24 ribozyme that lacks the linker region (U85–U90; see Fig. 1) is fully active in tRNA aminocacylation in trans (we refer to this ribozyme as r24Δ6) (16). Therefore, even though the above mapping results suggest the localization of Mg²⁺ at the C89 region, it is very unlikely that this Mg²⁺ plays a critical role in catalysis. Similarly, Tb³⁺ cleavages that occurred in the V-loop most likely represent general Mg²⁺-binding sites in tRNA rather than critical sites for catalysis, since this site is structurally distant from the reaction site. Thus, we focus our discussions on the L3 and J2a/3 regions to identify the critical Mg²⁺-binding sites for catalysis.

To see whether the Tb³⁺ cleavage sites can be conserved in the trans-acting ribozyme, we repeated the Tb³⁺ cleavage in the presence of 50 mM Mg²⁺ using r24Δ6 (Fig. 3C). At 1–0.05 mM concentrations of Tb³⁺ (lanes 1–3) cleavage occurred at A60–U62 and U67–U68, which is consistent with those observed for pre-24 (Fig. 3C versus A). These results agree with the previous observation that 50 μM Tb³⁺ is sufficient to cleave RNA. Based on our previous chemical and nucleotide analog interference mappings, it has been proposed that two regions, U59–U62 and U67–U68, most likely constitute the amino acid-binding site (Fig. 1). The consistent cleavage at A60, U68 and their neighboring bases observed in both cis- and trans-acting ribozymes suggests that Mg²⁺ ion(s) bound to these sites may play critical roles in forming the amino acid-binding site.

**Tb³⁺-mediated RNA cleavage in competition with Mg²⁺**

The above experimental results allowed us to set up the appropriate conditions to perform the competition assay in the reverse order. We thus mapped potential Mg²⁺-binding sites of pre-24 in the presence of 50 μM Tb³⁺ competing with 0–50 mM Mg²⁺ (Fig. 4A, lanes 1–5). Intensive cleavage occurred at G66 in the absence of Mg²⁺ along with weak cleavages in the A60 and U68 regions (lane 1). This intense cleavage sharply decreased with an increase in Mg²⁺ concentration, suggesting that this site overlaps the Mg²⁺-binding site(s). Earlier studies suggested that the U67–U68 and A60 regions are in close proximity to the amino acid-binding site (16). Based on the above observation, we propose that Mg²⁺ binds to the G66 site clustered with the U67–U68 and A60 regions, thus forming the amino acid-binding site (Fig. 4B). Although we also observed an intense cleavage at C89 along with the same gradual decrease in the cleavage profile with an increase in Mg²⁺ concentration, this Mg²⁺-binding region is not critical for the ribozyme activity, as previously discussed. Thus, we exclude this site from the consideration of catalytically significant metal-binding sites.

Interestingly, when the Mg²⁺ concentration was increased from 10 to 50 mM, the cleavage intensity at G73 gradually increased (lanes 3–5). Careful examination of the previous Tb³⁺ concentration-dependent cleavage profile in Figure 3A also indicated a similar increase in cleavage at G73 when the Tb³⁺ concentration was much lower than that of Mg²⁺ (Fig. 3A, lanes 3–5). Thus, the G73 cleavage was enhanced when an excess of Mg²⁺ was added to compete with a low concentration of Tb³⁺. Moreover, G73 and its adjacent bases were strongly cleaved in the Mg²⁺-dependent cleavage at high concentrations (Fig. 3B, lanes 1 and 2), suggesting that this site is also a potential Mg²⁺-binding site.

Taken together, two potential Mg²⁺-binding sites can be proposed (Fig. 4B). The first site is at G66, which is clustered with the U67–U68 and A60 regions to form a high affinity Mg²⁺-binding site (we refer to this high affinity metal-binding site as the G66 site; Fig. 4B, circles). This is supported by strong Tb³⁺ cleavage at this site in the absence or presence of a limited amount of Mg²⁺, and also by the complete inhibition of its cleavage in the presence of >10 mM Mg²⁺ (Fig. 4A, lanes 1–3). The Mg²⁺ binding to this site likely correlates with the rapid increase in activity observed in the region <10 mM Mg²⁺ (Fig. 2C), and the Hill analysis in this region suggests that a single Mg²⁺ is responsible for the activity. Thus, we propose a single Mg²⁺ binds to this high affinity metal-binding site.

The second Mg²⁺-binding site is at the bases neighboring G73, which is a low affinity metal-binding site (Fig. 4B, rectangle). This is supported by the induction of Tb³⁺ cleavage at G73 by the addition of 10–50 mM Mg²⁺ (Fig. 4A, lanes 3–5) and also by the strong Mg²⁺-dependent cleavage at the bases neighboring G73 at high Mg²⁺ concentrations (Fig. 3B). It should be noted that since G73 remains cleaved in the

![Figure 3. Tb³⁺ and Mg²⁺ concentration-dependent RNA cleavage. (A) Sites of Tb³⁺ cleavage in 5'-labeled pre-24. Reactions were carried out in the presence of 100 mM Mg²⁺, incubated at 25°C for 1 h (pH 7.5), then resolved by 12% denaturing PAGE. Lanes 1–6 represent samples incubated with Tb³⁺ at the following concentrations: 5, 1, 0.5, 0.1, 0.04 and 0 mM. (B) Sites of Mg²⁺ cleavage in 5'-labeled pre-24. Reactions were carried out at 25°C for 4 h (pH 9.0). Lanes 1–7 represent samples incubated with Mg²⁺ at the following concentrations: 200, 100, 50, 20, 10, 1 and 0.5 mM. (C) Sites of Tb³⁺ cleavage in 5'-labeled r24 that lacks U85–U90 (r24Δ6). Reactions were carried out in the presence of 0.5 mM 5'-labeled r24Δ6, 0.5 μM v1-tRNA and 50 mM Mg²⁺, incubated at 25°C for 1.25 h (pH 7.5). Lanes 1–3 represent samples incubated with Tb³⁺ at the following concentrations: 1, 0.1 and 0.05 mM.](image-url)
presence of high Mg\(^{2+}\) concentrations, G73 would not be directly responsible for metal binding. Based on Mg\(^{2+}\)-dependent cleavage data (Fig. 3B), we assume that the neighboring bases, U72 and/or C74, may constitute the metal-dependent cleavage sites, Co(NH\(_3\))\(_6^{3+}\) would not bind to the second metal-binding site. Upon Mg\(^{2+}\) binding to this site, Tb\(^{3+}\) accessibility to G73 increases, which results in cleavage. For simplicity, we refer to this low affinity metal-binding site as the G73 site.

The Co(NH\(_3\))\(_6^{3+}\) titration experiment shown in Figure 2C revealed that it can assist in catalysis only at low concentrations. This seems to illustrate the activity assisted by the first metal ion appearing in the Mg\(^{2+}\) titration. If this assumption is correct, in the context of two (or more than two) Mg\(^{2+}\)-binding sites, Co(NH\(_3\))\(_6^{3+}\) would not bind to the second metal-binding site (the G73 site). This prediction motivated us to perform Tb\(^{3+}\) cleavage competing with Co(NH\(_3\))\(_6^{3+}\) (Fig. 4A, lanes 6–11). As expected, even a very low concentration of Co(NH\(_3\))\(_6^{3+}\), e.g. 0.5 mM (lane 7), competes effectively with Tb\(^{3+}\) for the G66 site, whereas increasing the Co(NH\(_3\))\(_6^{3+}\) concentration does not induce cleavage at G73 (lanes 9–10). This is in sharp contrast to the observations for the Tb\(^{3+}\)–Mg\(^{2+}\) experiment (lanes 3–4). Thus, Co(NH\(_3\))\(_6^{3+}\) can bind to the G66 site and support catalysis, but it cannot fully assist catalysis due to outersphere coordination. We therefore hypothesize that metal ions bound to the G66 and G73 sites are outersphere and innersphere coordinated metals, respectively.

**Mutations in the high affinity metal-binding site**

In order to cleave the RNA phosphate backbone, Tb\(^{3+}\) generally coordinates to the 2'-OH group, which facilitates its in-line attack on the 3'-bridging phosphate. Thus, we can assume that one of the potential heteroatoms that interacts with Tb\(^{3+}\) in the high affinity metal-binding site is the 2'-OH of G66. Since G66 forms a wobble base pair with U79 (Fig. 1, left), this wobble geometry may be critical to make the 2'-OH of G66 accessible to Mg\(^{2+}\). To test this hypothesis, we mutated this base pair to Watson–Crick pairs, A66:U79 and G66:C79 (Fig. 4B). For convenience of the introduction of mutations into the ribozyme, we used the trans-acting r24Δ6 ribozyme and compared the tRNA-aminoacylation activities of these mutants with the wild-type.

As expected, both mutants exhibit reduced activities, while the G66A mutation shows a more drastic drop in activity than U79C (Fig. 5). We have previously used nucleotide analog interference mapping to determine important bases in pre-24; we observed strong interference at G66 by inosine substitution (16). These results suggest that in addition to the 2'-OH of G66, the guanine base may also be involved in the formation of the metal-binding site. We have also shown that site-specific mutation of the bases neighboring G66, U67 and U68, to the corresponding C (U67C or U68C) reduces the activity by 6- and 2-fold, respectively. Thus, U67 and U68 in concert with G66 are important for the formation of the amino acid-binding site as well as formation of the high affinity metal-binding site.

**Monovalent metal ions support ribozyme catalysis**

Substitution of Mg\(^{2+}\) by monovalent metal ions has recently been used to investigate the roles of metal ions in ribozymes (8–10,28–30). A critical difference between Mg\(^{2+}\) and monovalent ions is that Mg\(^{2+}\) can lower the pK\(_a\) of a coordinating OH group and facilitate the generation of a hydroxide or alkoxide ion, whereas monovalent ions can coordinate to the OH group, but they cannot lower its pK\(_a\). It should be noted that Co(NH\(_3\))\(_6^{3+}\) is another kind of metal ion that is unable to generate a hydroxide or alkoxide ion. However, because of its strong electropositivity, it can bind strongly to the electronegative environment of the metal-binding site(s) (31,32). Thus, when Co(NH\(_3\))\(_6^{3+}\) stimulates ribozyme catalysis, it is possible that the essential metal ions do not generate such a hydroxide or alkoxide ion, but play other roles, such as stabilizing the catalytically critical structure or neutralizing an oxyanion in the transition state. Because the catalytic activity of our ribozyme is assisted by Co(NH\(_3\))\(_6^{3+}\), we wondered whether monovalent ions can also support ribozyme catalysis.

We first compared yields of the aminoacylation product near the end point in the presence of 0.1 M Mg\(^{2+}\), two high concentrations of three monovalent ions, and no metal ions (Fig. 6A). Li\(^{+}\) and Na\(^{+}\) at 2.5 M supported catalysis, yielding nearly the same amount of aminoacyl product as observed for 0.1 M Mg\(^{2+}\). Li\(^{+}\) and Na\(^{+}\) at 1 M showed approximately half of...
Therefore, we would like to cautiously state that all Mg\(^{2+}\) ions observed for Mg\(^{2+}\) below 0.1 M (see also Fig. 2C), where the catalytic roles are not ruled out. Our previous studies of the cis-acting pre-24 and its mutants (G66A and U79C). Trans-aminocacylation was carried out in the presence of 2 μM ribozyme and 0.5 μM v1-tRNA, incubated with 5 mM biotin-Phe-CME at 25°C for 5 or 60 min (pH 7.5). a, aminocacylated v1-tRNA complexed with SAv; b, v1-tRNA.

DISCUSSION

Our previous studies of the cis-acting pre-24 and its trans-acting version r24Δ6 have revealed that the primary interaction of r24 with tRNA is the base pair interaction of G70–U72 with G73–C75, as shown in Figure 1 (15,16). Chemical and nucleotide analog interference mappings of pre-24 have also revealed the potential phenylalanine-binding site to be embedded in J2a/3 and U67–U68 (Fig. 1), although other undetermined residues may also participate. A series of biochemical experiments have revealed an interesting correlation of our previous knowledge on the structure–function relationship to the Mg\(^{2+}\)-binding sites and their roles in the formation of the catalytic core (Fig. 4B). The high affinity Mg\(^{2+}\) is recruited to the A60 and G66–U68 region, referred to as the G66 site (Fig. 4B). This metal-binding site is in close proximity to the Phe-binding site, and thus Mg\(^{2+}\) likely plays a critical role in forming the amino acid-binding site. The activity observed at 2.5 M. K\(^{+}\) also supported catalysis, but the degree of activity was much less than the other two cations. We also determined the rate constants in the presence of 2.5 or 2.3 M monovalent metal ions in the presence of 5 mM amino acid substrate (Fig. 6A). Again, the observed rate constants for 2.5 M Li\(^{+}\) and Na\(^{+}\) were comparable to that for 0.1 M Mg\(^{2+}\), whereas 2.3 M K\(^{+}\) supported catalysis considerably less well than the other cations.

To gain more insight into the concentration dependence of monovalent ion-assisted catalysis, Na\(^{+}\) was chosen for the titration experiment (Fig. 6B). Below 0.5 M Na\(^{+}\) the activity was barely observed, but above 1 M the activity increased to an appreciable level. The activity appeared to linearly increase in the range 1–2.5 M. Due to the limited number of data points, we cannot accurately assess the cooperativity value for Na\(^{+}\). However, based on a comparison with the titration curve observed for Mg\(^{2+}\) below 0.1 M (see also Fig. 2C), where the Hill coefficient is nearly 1 (Fig. 2D), such a value for Na\(^{+}\) seems much greater than 1. This argues that multiple Na\(^{+}\) ions are required to display an activity comparable to the Mg\(^{2+}\)-assisted catalysis occurring below 0.1 M. Thus, even though Na\(^{+}\) can assist the ribozyme activity at high concentrations, it is difficult to conclude that the catalytic mechanism assisted by Na\(^{+}\) is the same or similar to that by Mg\(^{2+}\). Therefore, we would like to cautiously state that all Mg\(^{2+}\) ions bound to this ribozyme likely play structural roles, but catalytic roles are not ruled out.

G66:U79 wobble base pair is also involved in constituting the high affinity metal-binding site. Based on the results from the Co(NH\(_3\))\(_6\)\(^{3+}\) titration and its competition with Tb\(^{3+}\), it is assumed that the Mg\(^{2+}\) bound to the G66 site has a hexahydrated form, and thus interacts with the residues of the G66–U68 cluster through outersphere water ligands.

On the other hand, the second Mg\(^{2+}\)-binding site, which is a low affinity metal-binding site, is most likely located at U72–C74, referred to as the G73 site. Due to the low affinity property of this site, Mg\(^{2+}\) can bind to this site only at high concentrations (>50 mM), which further enhances the catalytic activity of the ribozyme. Mg\(^{2+}\) binding to this site may cause a minor structural change at this site, which increases the accessibility to Tb\(^{3+}\), resulting in the observed cleavage at G73 (Fig. 4A, lane 5). This site can be occupied only by inner sphere metal ions and, therefore, Co(NH\(_3\))\(_6\)\(^{3+}\) is unable to enhance the activity like Mg\(^{2+}\) does. This site is in close proximity to the tRNA-binding site. We thus propose that the second metal ion(s) plays a structural role to stabilize the ribozyme–tRNA complex, but we cannot rule out the possibility that it assists the catalysis in concert with the first metal ion in the active site.
Despite a number of experiments performed on this riboyme system, the absolute roles of Mg$^{2+}$ ions have not yet been defined clearly. However, by virtue of the observations that monovalent ions can support catalysis at high concentrations and Co(NH$_3$)$_6^{3+}$ can substitute for the role of the first Mg$^{2+}$, we can rule out that the high affinity metal ion generates a hydride or alkoxide ion during catalysis. Unfortunately, because the mechanism assisted by monovalent ions likely differs from that assisted by Mg$^{2+}$ and Co(NH$_3$)$_6^{3+}$, we cannot rule out a catalytic role of metal ions. For instance, the metal ion bound to the G66 site may play a role in neutralizing an oxyanion of the acyl intermediate that appears in the transition state, since its location is in close proximity to the G70–U72 tRNA-binding site that brings the 3′-adenosine of tRNA to the G66 site. Further detailed studies are required to gain insights into the mechanism of this riboyme-catalyzed reaction. Nonetheless, our biochemical studies reported here provide new evidence that the high affinity Mg$^{2+}$ plays a role in bringing the J2a3 and L3 regions together and forming the Phe-binding site. Thus, we conclude that two or more Mg$^{2+}$ ions bound to the G66 and G73 sites likely play structural roles, but we cannot rule out the possibility that Mg$^{2+}$ in the G66 site may also play a catalytic role in neutralizing the oxyanion in the transition state.

We previously utilized a similar approach to investigate metal-binding sites of a GlnRS riboyme, called AD02 (21). Our studies have revealed that it also has two metal-binding sites, where the high affinity Mg$^{2+}$-binding site can be filled with Co(NH$_3$)$_6^{3+}$, but the low affinity site cannot. Binding of Mg$^{2+}$ to both sites is necessary to promote catalysis, unlike r24. Moreover, even though Co(NH$_3$)$_6^{3+}$ can bind to the high affinity site, it fails to stimulate AD02 catalysis, but acts as a strong inhibitor for catalysis. This suggests that in AD02, inneresphere coordination of Mg$^{2+}$ to either or both sites is important for activity, and the metal ions are necessary to constitute the Gln-binding site. These two ARS-like ribozymes, r24 and AD02, use the same activating group (the cyanoethyl ester), although they recognize the distinct side chains, Phe and Gln, with remarkable specificity. Interestingly, the coordination mode of Mg$^{2+}$ required for these ribozymes is different, yet the metal ions in both ribozymes play a critical role in constituting the amino acid-binding site and promoting catalysis. Thus, regardless of the exact roles of metal ions in these ARS-like ribozymes (i.e. catalytic or structural), Mg$^{2+}$ is essential to exhibit their maximal activity.

This report represents an approach to probe the potential metal ion-binding sites and their roles in the riboyme using a combination of three techniques: (i) metal-dependent hydrolytic cleavage; (ii) competition of Mg$^{2+}$ and Co(NH$_3$)$_6^{3+}$ against Tb$^{3+}$ under optimized conditions; (iii) metal-dependent kinetics of wild-type and mutants with Mg$^{2+}$, Co(NH$_3$)$_6^{3+}$ and monovalent ions. The combination of these techniques is a powerful tool for rapid identification of the potential metal-binding sites and roles of metal ions in catalysis and is also applicable to many artificial ribozymes (21,22,28), as well as naturally occurring ribozymes (26,27).

In conclusion, we have demonstrated the roles of inner- and outersphere coordinated metal ions in the PheRS riboyme. An outersphere coordinated metal ion binds to the G66 site with high affinity. Since this G66 site is a part of the phenylalanine recognition motif, this metal ion most likely plays a critical role in forming the amino acid-binding site. We have also shown that an innerphere coordinated metal ion binds to G73 site, which is adjacent to the tRNA recognition motif (G70–U72). Thus, this metal may induce a conformational change at the G73 site and stabilize the riboyme–tRNA complex, which further enhances the activity. Most importantly, Mg$^{2+}$ ions, which bind these two sites in different coordination modes (innerphere and outersphere), are critical for the PheRS riboyme to exhibit maximum activity.

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