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Accommodation of Ca(II) ions for catalytic activity by a group I ribozyme

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Abstract

The wildtype *Tetrahymena* ribozyme cannot catalyze detectable levels of phosphotransfer activity *in vitro* on an exogenous RNA substrate oligonucleotide when calcium(II) is supplied as the only available divalent ion. Nevertheless, low-error mutants of this ribozyme have been acquired through directed evolution that do have activity in 10 mM $CaCl_2$. The mechanisms for such Ca(II) accommodation are not known. Here, we assayed the entire molecule in an effort to identify the roles of the mutations in allowing catalytic activity in Ca(II). We used four biochemical probing techniques – native-gel electrophoresis, hydroxyl radical footprinting, terbium(III) cleavage footprinting, and phosphorothioate interference mapping – to compare the solution structure of the wildtype ribozyme with that of a Ca(II)-active five-site mutant. We compared the gross folding patterns and specific metal-binding sites in both $MgCl_2$ and $CaCl_2$ solutions. We detected no large-scale folding differences between the two RNAs in either metal. However, we did discover a limited number of local folding differences, involving regions of the RNA affected by positions 42, 188, and 270. These data support the notion that Ca(II) is accommodated by the *Tetrahymena* ribozyme by a slight breathing at the active site, but that alterations at, near to, and distal from the active site can all contribute to Ca(II)-based activity.

Keywords: Catalytic RNA; Ribozyme; Group I; *Tetrahymena*; Calcium

1. Introduction

Most RNA enzymes are metalloenzymes, exploiting the potential of divalent cation cofactors at their active sites to enhance their catalytic functions [1–3]. Magnesium is by far the preferred divalent cation, used by almost every natural ribozyme to promote folding and/or catalysis [4–6]. The properties of this cation, such as its small size and consequent high charge density, coordination geometry, moderate pK_a values of its aqua ion, and bioavailability all contribute to its suitability as an RNA cofactor. While it was realized long ago that the divalent cationic nature of Mg(II) was essential to provide the electrostatic shielding necessary for RNAs to adopt 2° and 3° structures, only in the last 15 years or so have we appreciated the complexity of the myriad roles that Mg(II) can play in RNA function. For example, we now realize that Mg(II) ions can bind to RNA in multiple fashions, not only in terms of the variety of inner-sphere *vs.* outer-sphere coordination arrangements, but also in terms of specific, localized, and diffuse (polyelectrolytic) locations along the RNA backbone [7–9]. In fact, the number and locations of divalent cations in the catalytic centers of some ribozymes has been the subject of a vigorous debate. Understanding how ribozymes can accommodate a broader range of divalent cations may help clarify some of these important issues [9–11].

Group I ribozymes, like other large (>150 nt) catalytic RNAs, have a strong dependence on Mg(II) for proper activity. These ribozymes can catalyze phosphotransfer reactions when Mg(II) is replaced by Mn(II) but not any other divalent, including Ca(II). The naturally occurring

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forms of the *Tetrahymena* and *Azoarcus* ribozymes display no detectable activity *in vitro* when supplied with Ca(II) as the only available divalent [12–14]. Furthermore, kinetic data indicate that even low concentrations of the Ca(II) ion actually *inhibit* the chemical step of endoribonuclease activity of the *Tetrahymena* ribozyme [15]. However, through evolution *in vitro*, variants of the *Tetrahymena* ribozyme have been discovered (Fig. 1) possessing significant amounts of activity in 10 mM $CaCl_2$ [16,17]. A particular 5-nucleotide-site mutant has a catalytic efficiency (k_{cat}/K_m) in Ca(II) that is approximately 10^5-fold less than that of the wildtype in Mg(II). Yet the activity of this mutant in Ca(II) is not trivial: it can catalyze the conversion of almost half as many moles of its substrate (an RNA oligomer) into product as can the wildtype in Mg(II) under their respective optimal *in vitro* reaction conditions [16].

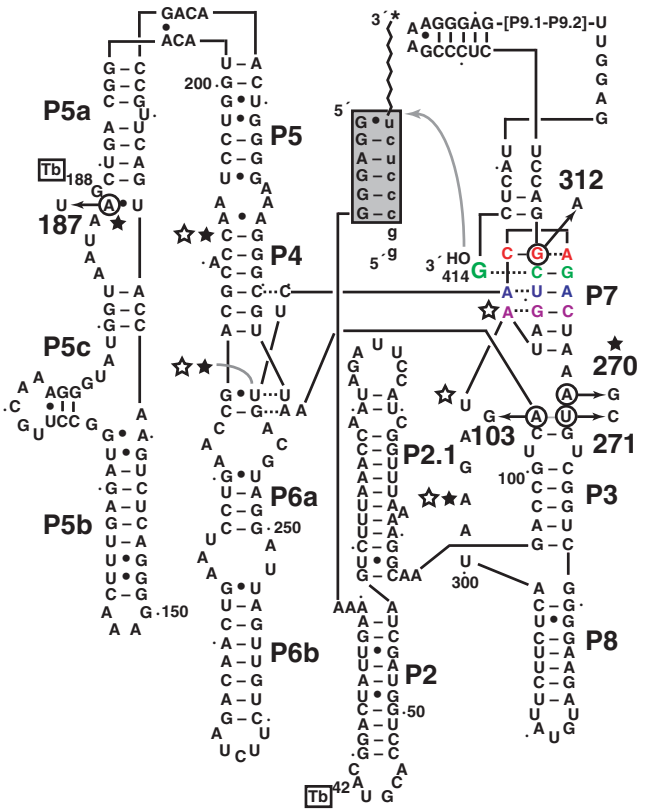

Fig. 1. The *Tetrahymena* ribozyme and proposed binding sites for catalytically critical divalent cations. The nucleotide sequence of the wildtype is shown, with the five positions that can be altered to engender the ribozyme competent to perform catalysis in 10 mM $CaCl_2$ indicated by circles, with arrows to their respective mutations. Nucleotides 42 and 188 are those that reveal altered Tb(III) cleavage patterns when the wildtype is compared to the mutant. Closed and open stars represent pro-R_p oxygens with strong evidence of binding critical Ca(II) and Mg(II) ions, respectively, from this and other phosphorothioate interference mapping studies. Nucleotides in color comprise the catalytic core, with dashed lines representing Hoogsteen–WC or Hoogsteen–Hoogsteen hydrogen bonds that contribute to base-triple interactions [22]. Shaded box is the internal guide sequence, with substrate for the *in vitro* phosphorotransfer reaction indicated in lower-case letters and wavy line.

While the activity of this mutant is robust, it is still not known what molecular mechanism is used to manifest Ca(II)-dependent RNA cleavage. It is conceivable that these five mutations significantly remodel the global folded conformation of the molecule to allow redesign of the active site in such a way that a different catalytic strategy is available for Ca(II) ions. Yet small angle X-ray scatter data [18] and native-gel data [19] both demonstrate that the native fold in Ca(II) is only slightly larger than that in Mg(II). Therefore, it is unlikely that the molecule folds in Ca(II) in a fashion that differs significantly from its conformation in Mg(II). If true, Ca(II)-dependent activity is more likely achieved merely by a slight expansion of the active site to accommodate the Ca(II) ion, although this has never been explicitly tested. Ca(II) differs from Mg(II) in that its radius is 33–39% larger (ionic radius of 0.99 Å *vs.* 0.72 Å for Mg(II), and a hexahydrated radius of 1.14 Å *vs.* 0.86 Å for Mg(II)). In addition, the pK_a of the coordinated waters of Ca(II) is about 12.9, a full 1.5 log units higher than that of Mg(II), and the Ca(II) ion has more potential to take on an expanded coordination sphere beyond solely hexavalent. It is unknown how these differences influence biochemical adaptation of ribozymes to Ca(II).

Since the selections for Ca(II) accommodation, a combination of X-ray crystallography data [20–22] and site-directed nucleotide analog interference mapping data [23–26] has allowed the construction of models of the active site of the wildtype *Tetrahymena* ribozyme in Mg(II). One prominent model positioned three Mg(II) ions at the catalytic center of the molecule held in place via coordination to non-bridging phosphate oxygens (NBPOs) of both the substrate oligoribonucleotide and of the ribozyme itself [26,27], although more recent data suggests that two Mg(II) ions can satisfy most of the active-site ligands (cf. [22]). In fact, most available data are consistent with the hypothesis that all group I introns may utilize the same two-metal-ion mechanism for splicing [28,29].

In any event, the pro-S_p NBPOs of A207, C208, U305, and A306, the pro-R_p NBPOs of C262 and A306, and the 2′-OH of the G nucleophile appear to play dominant roles in the coordination of any active-site Mg(II) ions. Adjacent residues may also contribute to catalytic Mg(II) binding. If active-site expansion were all that is needed to engender activity in Ca(II), mutations at these residues and/or their neighbors might be sufficient. However, the mutations in the selected Ca(II)-competent ribozyme occur at positions 103, 187, 270, 271, and 312, none of which coincides with the residues identified above, making a prediction of how minor changes could lead to Ca(II) accommodation less straightforward. Here, we examine the roles of all positions in the RNA by probing the overall folds of the wildtype and mutant RNAs in the two metals and by querying the responses of the RNA to two types of metal-binding assays that provide information on localized folding processes.