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Comparison of the hammerhead cleavage reactions stimulated by monovalent and divalent cations

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ABSTRACT

Although the hammerhead reaction proceeds most efficiently in divalent cations, cleavage in 4 M LiCl is only ~10-fold slower than under standard conditions of 10 mM $MgCl_2$ (Murray et al., *Chem Biol*, 1998, *5*:587–595; Curtis & Bartel, *RNA*, 2001, this issue, pp. 546–552). To determine if the catalytic mechanism with high concentrations of monovalent cations is similar to that with divalent cations, we compared the activities of a series of modified hammerhead ribozymes in the two ionic conditions. Nearly all of the modifications have similar deleterious effects under both reaction conditions, suggesting that the hammerhead adopts the same general catalytic structure with both monovalent and divalent cations. However, modification of three ligands previously implicated in the binding of a functional divalent metal ion have substantially smaller effects on the cleavage rate in Li^+ than in Mg^{2+}. This result suggests that an interaction analogous to the interaction made by this divalent metal ion is absent in the monovalent reaction. Although the contribution of this divalent metal ion to the overall reaction rate is relatively modest, its presence is needed to achieve the full catalytic rate. The role of this ion appears to be in facilitating formation of the active structure, and any direct chemical role of metal ions in hammerhead catalysis is small.

Keywords: biological catalysis; enzyme; mechanistic analysis; metal ions; RNA cleavage; thio effects

INTRODUCTION

The hammerhead ribozyme is a self-cleaving RNA motif found in several plant satellite RNA and viroid genomes (Forster & Symons, 1987a, 1987b; Uhlenbeck, 1987; Fig. 1). It catalyzes an intramolecular phosphoryl transfer reaction to generate two products, one with a 2′-3′ cyclic phosphate and the other with a 5′-hydroxyl. The hammerhead has been modified to develop an experimentally accessible, kinetically well-defined system in which to study catalysis (e.g., Uhlenbeck, 1987; Hertel et al., 1994, 1998; Stage-Zimmermann & Uhlenbeck, 1998). The in vitro reaction is most efficient in millimolar quantities of divalent metal cations and displays a rate order dependency on one metal ion. These observations are consistent with the view that RNA catalysis might generally involve metal ions (Dahm et al.,

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Abbreviations: BTP, bis-tris[hydroxymethyl]-aminomethane; E•S: ribozyme•substrate complex; E: generic references to ribozyme; HH8 and HH16: hammerhead ribozymes 8 and 16, respectively (Fig. 1); HPLC, high-performance liquid chromatography; Na_2EDTA, disodium ethylenediaminetetraacetate; NaHEPES, sodium N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonate; NaMES, sodium 2-[N-morpholino]ethanesulfonate; NaMOPS: sodium 3-(N-morpholino)-propanesulfonate; NaPIPES, sodium piperazine-N,N′-bis[2-ethanesulfonate]; S: generic references to oligonucleotide substrate; wt: unmodified ribozyme. Hammerhead variants are described by acronyms analogous to protein mutations; e.g., C3X refers to a ribozyme with an abasic residue at position 3.

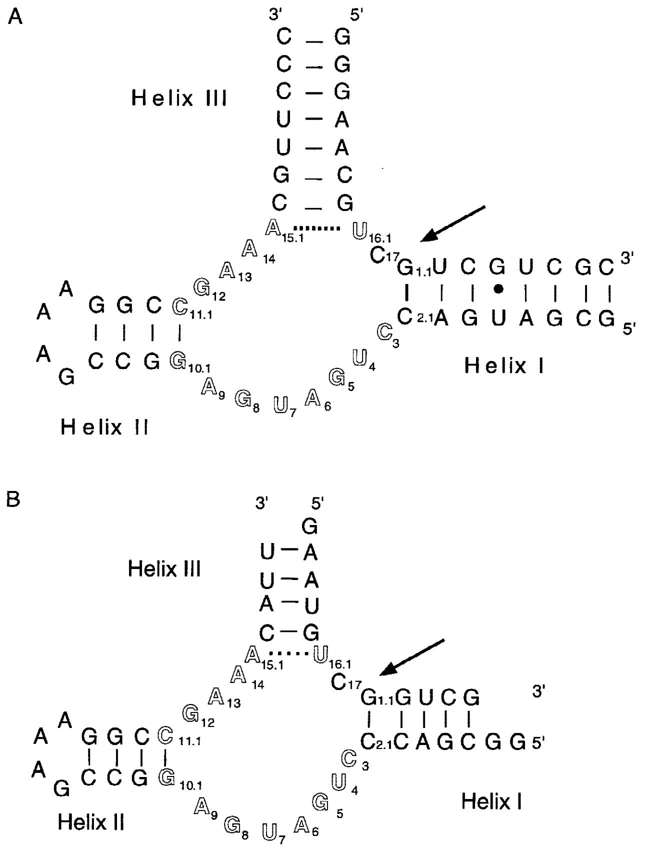

FIGURE 1. Hammerhead ribozymes and substrates. Secondary structure of HH16 (**A**) and HH8 (**B**) with their respective substrates. Numbering is according to Hertel et al. (1992). The catalytic core is shown in outlined letters, and the arrow depicts the cleavage site P1.1 phosphoryl group.

1993; Pan et al., 1993; Steitz & Steitz, 1993; Yarus, 1993; Pyle, 1996; Narlikar & Herschlag, 1997; Feig & Uhlenbeck, 1999).

More recent experiments, however, have provided strong evidence that many ribozymes can perform catalysis by mechanisms not involving divalent metal ions. The hairpin ribozyme can carry out efficient catalysis in the absence of divalent metal ions and in the absence of direct metal ion coordination (Hampel & Cowan, 1997; Nesbitt et al., 1997; Young et al., 1997; Fedor, 2000). It was shown that the hammerhead and the VS ribozymes are active in the absence of divalent metal ions provided that very high concentrations of monovalent cations are present (Murray et al., 1998a). Several in vitro selection experiments have also successfully obtained RNA and DNA catalysts that operate in the apparent absence of divalent metal ions or their direct coordination (e.g., Geyer & Sen, 1997; Jayasena & Gold, 1997; Roth & Breaker, 1998; Suga et al., 1998). The X-ray crystal structure of the hepatitis delta ribozyme revealed a conserved base held at the position of strand cleavage (Ferre-D'Amare et al., 1998), and subsequent functional studies provided the first mechanistic evidence for general acid–base catalysis mediated by an RNA base, a protonated C residue (Perrotta et al., 1999; Nakano et al., 2000). Most recently, the X-ray crystal structure of the 50S ribosomal subunit has revealed that there are no protein residues in the vicinity of the peptidyl transfer active site, and a structure with a bound transition state analog provides no indication of any divalent metal ions bound in the active site (Ban et al., 2000; Nissen et al., 2000).

Despite investigation of more than a hundred site-specific modifications and several X-ray crystal structures (Pley et al., 1994; Scott et al., 1995, 1996; McKay, 1996; Murray et al., 1998b, 2000; Vaish et al., 1998; Zhou & Taira 1998), the mechanism of catalysis by the hammerhead ribozyme has remained obscure (McKay, 1996). Based upon the high rate of hammerhead cleavage in the presence of divalent metal ions, the differences in rate with different divalent metal ions, and competition experiments with different divalent metal ions, direct catalytic roles for divalent metal ions in the hammerhead reaction have been proposed (Taira et al., 1990; Perreault et al., 1991; Dahm et al., 1993; Scott & Klug, 1996; Pontius et al., 1997; Lott et al., 1998; Zhou & Taira, 1998). This conclusion received support from experiments showing that catalytically impaired hammerhead ribozymes containing phosphorothioate substitutions at certain locations could be "rescued" by the addition of thiophilic metal ions, which suggested that these phosphoryl groups participate in functional divalent metal ion interactions (Dahm & Uhlenbeck, 1991; Koizumi & Ohtsuka, 1991; Slim & Gait, 1991; Knoll et al., 1997; Peracchi et al., 1997; Scott & Uhlenbeck, 1999; Wang et al., 1999; Yoshinari & Taira, 2000). However, as specifically bound metal ions are typically critical for promoting the correct folding of RNA (Draper, 1996), it is difficult to rigorously distinguish direct participation of metal ions from such structural effects. Indeed, it has recently been proposed that the two rescuable phosphorothioate sites in the hammerhead coordinate a single metal ion whose primary role is to stabilize a form of the ribozyme that forms only transiently prior to catalysis (Wang et al., 1999).

The observation that a substantial amount of hammerhead catalysis occurs in the presence of high concentrations of monovalent cations and exchange–inert metal ions of higher valency (Murray et al., 1998a; Curtis & Bartel, 2001) provided an opportunity to distinguish the structural and catalytic roles of divalent metal ions in the reaction. However, before this could be done, it was critical to show that the hammerhead catalytic mechanism is the same in divalent and monovalent cations. We have compared the effect of a number of mutations and modifications of the hammerhead on the rate of cleavage in the presence of monovalent and divalent cations. The results support the previously proposed role of divalent metal ions in stimulating adoption of the active conformation of the hammerhead ribozyme and also suggest that any direct role of divalent metal ions in the reaction chemistry is not large.

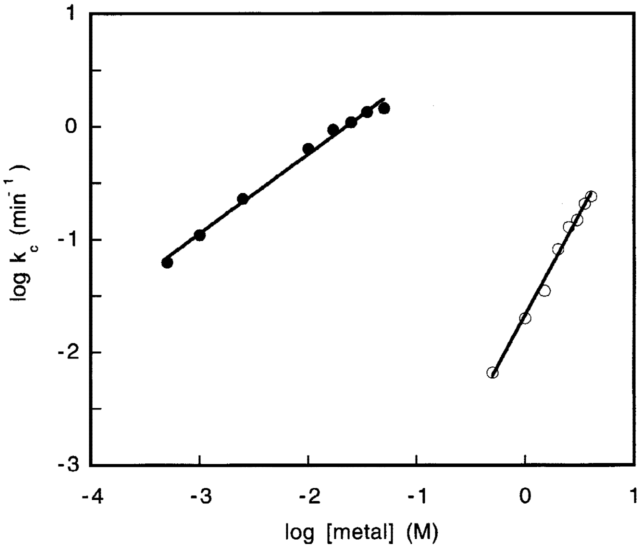

FIGURE 2. Concentration dependence for hammerhead catalysis in the presence of Mg^{2+} (●) or Li^+ (○). The lines are drawn as a linear best fit with the slopes of 0.7 and 1.8 for reactions in Mg^{2+} and Li^+, respectively. Reaction conditions: 50 mM NaPIPES, pH 7.5, 1 μM HH8, trace substrate, 0.1 mM Na_2EDTA, and varying concentrations of metal chloride salt.

RESULTS AND DISCUSSION

Hammerhead cleavage in monovalent cations

Two kinetically and thermodynamically well-characterized hammerhead ribozyme constructs, HH8 and HH16 (Fig. 1) were used in this study (Fedor & Uhlenbeck, 1992; Hertel et al., 1994, 1998). Previous studies have shown that effects of modifications are comparable between the two ribozymes (Baidya & Uhlenbeck, 1997; Baidya et al., 1997). Reactions were performed under single-turnover conditions, with ribozyme saturating with respect to the oligonucleotide substrate to ensure that conversion of the E•S complex to E•P was observed kinetically without complications from slow product release. To compare hammerhead catalysis stimulated by divalent and monovalent cations, the cleavage of HH8 as a function of cation concentration is shown in Figure 2. Consistent with a previous study, HH8 displays an approximately first-order dependence on Mg^{2+} concentration until saturation at a rate of 1.3 min^{-1} is achieved (Dahm & Uhlenbeck, 1991).[6]

In contrast to the efficient cleavage in millimolar Mg^{2+}, the hammerhead cleavage rate is very slow in LiCl below 0.5 M. The reaction rate shows a second-order dependence on Li^+ up to 4 M where a rate of 0.24 min^{-1} is reached. No saturation is observed for the reaction in Li^+, which may reflect much weaker binding of Li^+ or the nonideal behavior of concentrated LiCl solutions. The observed difference in the ion concentration dependencies of hammerhead cleavage rates may reflect the differential ability of monovalent and divalent cations to interact with and neutralize the charge in the polyanionic ribozyme•substrate complex.

To further characterize hammerhead cleavage in monovalent cations, the pH dependence of the reaction was examined. The pH-rate profile of HH16 cleavage was determined in the presence of 4 M Li^+ and 4 M NH_4^+ and compared to that in 10 mM Mg^{2+}. As had been found previously for many hammerheads in the presence of various divalent cations (Dahm et al., 1993; Stage-Zimmermann & Uhlenbeck, 1998; Zhou & Taira 1998), a log-linear pH-rate profile with a slope of one was observed (data not shown). As observed previously (Murray et al., 1998a; Curtis & Bartel, 2001), the rates of the monovalent-stimulated reactions were only 10- to 100-fold below those measured in 10 mM Mg^{2+} (Fig. 2; data not shown). The data displayed more scatter in the case of the monovalent-stimulated pH-rate profiles than in the case of the Mg^{2+} dependent reactions, which may reflect differential effects of the high salt concentrations on the activity of buffer components for the different buffers used. The pH dependencies indicate that the hammerhead reaction requires loss of a single proton prior to the rate-limiting step, whether it is stimulated by either monovalent or divalent cations. The slope of one for the NH_4^+ reaction is particularly significant as it rules out the involvement of NH_3, which has the potential to act as a general base. Catalysis stimulated by NH_4^+ then indicates that hammerhead cleavage can occur in the absence of any metal ions (Murray et al., 1998a).

Cleavage of modified hammerhead ribozymes in Mg^{2+} and Li^+

The cleavage rates of a series of previously characterized modified hammerhead ribozymes were compared in 10 mM Mg^{2+} and 4 M Li^+. If all the modifications were to have analogous effects under both conditions, the data would imply a common reaction mechanism, one presumably not directly utilizing divalent metal ions for catalysis. However, any differences in Mg^{2+} and Li^+ could highlight differences in the two reaction mechanisms. Thirteen derivatives of HH16 containing single abasic sites throughout the catalytic core (Peracchi et al., 1996, 1998) and six derivatives of HH8 containing modifications at C3, G5, and C17 (Baidya et al., 1997) were measured in Mg^{2+} and Li^+ (Table 1). In all cases the

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[6] Although many hammerhead ribozymes show a first-order dependence on Mg^{2+} concentration, the values of K_{Mg} and maximal rate constant vary considerably among different sequences (Clouet-D'Orval & Uhlenbeck, 1997; Stage-Zimmermann & Uhlenbeck, 1998) suggesting that these properties are not intrinsic to the hammerhead core. For example, it is possible that the reaction rate levels off due to the binding of an inhibitory Mg^{2+} ion and not because of saturation of the stimulatory ion(s). We therefore do not use these values in mechanistic interpretations.

TABLE 1. Effect of hammerhead mutations and modifications on reactions in divalent and monovalent cations[a]

		Metal cation			
		10 mM $MgCl_2$		4 M LiCl	
	Ribozyme	k_c (min^{-1})	k_{rel}[b]	k_c (min^{-1})	k_{rel}[b]
A. HH16	WT	0.43	(1)	8.9×10^{-2}	(1)
	C3X	$\leq 3 \times 10^{-6}$	$\leq 7 \times 10^{-6}$	4.4×10^{-6}	4.9×10^{-5}
	U4X	2.8×10^{-2}	6.5×10^{-2}	1.2×10^{-2}	0.14
	G5X	$\leq 3 \times 10^{-6}$	$\leq 7 \times 10^{-6}$	2.5×10^{-5}	2.8×10^{-4}
	A6X	7.3×10^{-6}	1.7×10^{-5}	4.0×10^{-5}	4.5×10^{-4}
	U7X	6.8×10^{-3}	1.6×10^{-2}	1.2×10^{-3}	1.3×10^{-2}
	G8X	$\leq 3 \times 10^{-6}$	$\leq 7 \times 10^{-6}$	3.9×10^{-6}	4.4×10^{-5}
	A9X	2.9×10^{-4}	6.7×10^{-4}	1.8×10^{-3}	0.02
	G10X	3.1×10^{-2}	7.2×10^{-2}	0.21	2.3
	C11X	7.6×10^{-2}	0.18	0.71	7.9
	G12X	7.8×10^{-5}	1.8×10^{-4}	1.0×10^{-3}	0.01
	A13X	$\leq 3 \times 10^{-6}$	$\leq 7 \times 10^{-6}$	2.2×10^{-5}	2.5×10^{-4}
	A14X	$\leq 3 \times 10^{-6}$	$\leq 7 \times 10^{-6}$	3.9×10^{-5}	4.4×10^{-4}
	A15X	$\leq 3 \times 10^{-6}$	$\leq 7 \times 10^{-6}$	4.5×10^{-6}	4.9×10^{-5}
B. HH8[c]	WT	1.4	(1)	0.18	(1)
	C17A	0.18	0.13	2.5×10^{-2}	0.14
	C17U	0.16	0.11	0.13	0.72
	C17Iso-C	3.7×10^{-4}	3.0×10^{-4}	2.2×10^{-4}	1.2×10^{-3}
	C17Phe	0.53	0.38	0.06	0.33
	C3U	4.0×10^{-4}	3.0×10^{-3}	4.0×10^{-4}	2.2×10^{-3}
	G5C	5.0×10^{-5}	4.0×10^{-5}	3.0×10^{-5}	1.6×10^{-4}

[a] All reactions were single turnover with a saturating excess of ribozyme relative to substrate and were carried out at 25 °C (see Methods). The rate constant k_c represents the reaction of the E•S complex. Reactions of HH16 and its variants were carried out with 200 nM ribozyme in 50 mM NaMOPS, pH 7.3, and reactions of HH8 and its variants were carried out with 1 μM ribozyme in NaPIPES, pH 7.5. "≤" indicates an upper limit for the reaction rate.

[b] $k_{rel} = k_c^{variant}/k_c^{wt}$.

[c] Errors were typically ≤30%.

rate constants and effects of the modifications (k_{rel}) observed in Mg^{2+} are the same within error (approximately twofold) as observed previously (Peracchi et al., 1996; Baidya et al., 1997).

Figure 3 graphically compares the relative rate constants for the 19 hammerhead variants under the two conditions. There is, in general, a striking correspondence between the effect of the modifications in Mg^{2+} and Li^+. For example, C3X, A6X, and G8X, which have undetectable catalysis in Mg^{2+}, are down $\gtrsim 10^4$-fold in Li^+, and U4X and U7X, which have only modest defects in Mg^{2+}, have similar modest defects in Li^+. Similarly, modification of C17 to A, U, or a phenyl group gives rate effects of less than 10-fold in both Mg^{2+} and Li^+, whereas modification to iso-C gives a substantially larger effect under both conditions. The overall correspondence between the effects of modifications in Mg^{2+} and Li^+ as observed in Figure 3 would be highly unlikely to arise from random chance. The results most simply suggest an analogous reaction mechanism in Mg^{2+} and Li^+.

There are, however, two differences between the Mg^{2+} and Li^+ data in Figure 3. First, the modifications with the largest effects in Mg^{2+} ($k_{rel} > 10^5$-fold) each have somewhat smaller effects in Li^+ ($k_{rel} \approx 10^4$-fold). This difference is not due to a faster background rate of cleavage in Li^+. In 50 mM NaPIPES, pH 7.0, the background rate of cleavage for the substrate was 2×10^{-6} min^{-1} in 4 M LiCl, which is similar to that in 10 mM Mg^{2+} (data not shown; Curtis & Bartel, 2001). We speculate that formation of the global catalytic conformation for hammerhead catalysis is less cooperative in Li^+ than in Mg^{2+}, resulting in smaller effects from individual modifications (see Peracchi et al., 1998, for discussion of cooperativity in formation of the active hammerhead conformation).

An interesting additional difference between the rate effects of the mutations studied in Mg^{2+} and in Li^+ concerns the substitution of abasic residues at positions G10.1 and C11.1 in HH16 (Fig. 3A). For these two mutant ribozymes, the cleavage rates in Li^+ were faster than that observed for the wild-type ribozyme. This finding contrasts with the modest rate decreases observed for these two hammerhead variants in Mg^{2+}. The crystal structure of the hammerhead ribozyme shows that residues G10.1 and C11.1 form a Watson–Crick base pair. Furthermore, the N7 of G10.1 has been implicated by both X-ray crystallographic analysis (Pley et al., 1994; Scott et al., 1996) and functional data (Peracchi et al., 1997; Wang et al., 1999) as a ligand for the binding of a divalent metal ion. We therefore tested the effect of removal of this nitrogen atom in Mg^{2+} and in Li^+, using

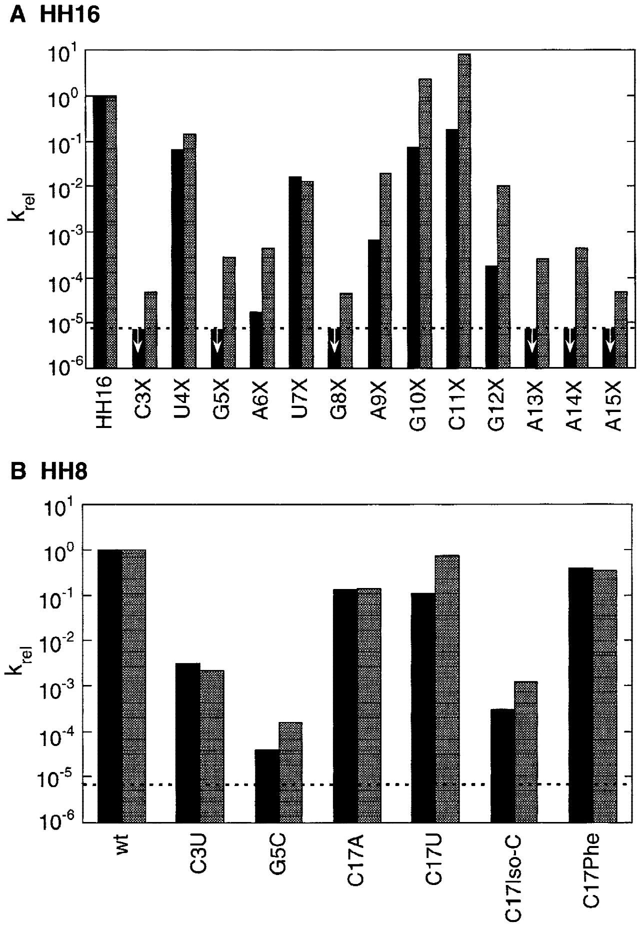

FIGURE 3. Comparison of the effect of mutations and modifications on reactions in 10 mM Mg^{2+} (solid bars) or 4 M Li^+ (gray bars). Wild-type (wt) and variant HH16 effects are shown in **A** and HH8 wt and variants in **B**. k_{rel} represents the reaction rate for the variant relative to wt under the same reaction conditions, such that $k_{rel} \equiv 1$ for wt in both Mg^{2+} and in Li^+. The dotted line defines the lower limit of detection; arrows represent upper limits for rate constants for specific ribozymes. Data are from Table 1.

the base 7-deazaG at position 10.1.[7] This site-specific change mimicked the effect of base ablation at G10.1, giving k_{rel} less than one in Mg^{2+} and greater than one in Li^+ (k_{rel} = 0.025 and 2.2, respectively; data not shown).

These results suggest that the hammerhead reaction in the presence of monovalent and divalent cations is analogous overall, but raise the possibility that hammerhead interactions that coordinate a stimulatory divalent cation may be absent in the reaction stimulated by monovalent cations. This finding prompted an examination of other hammerhead modifications known to affect binding of the divalent metal ions.

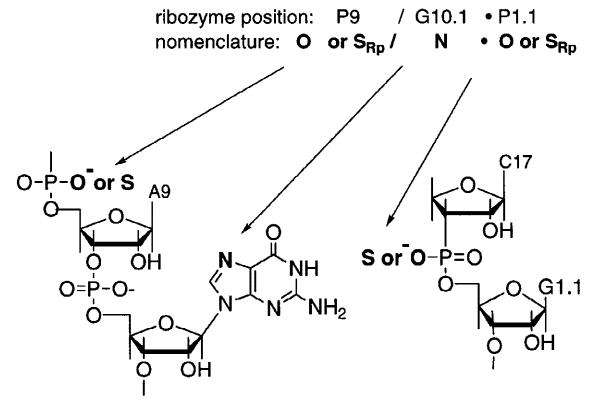

FIGURE 4. Nomenclature and depiction of sites of sulfur substitution at hammerhead phosphoryl oxygen atoms implicated in divalent metal ion binding on reactions, following Wang et al. (1999).

Thio effects in Mg^{2+} and Li^+ stimulated hammerhead catalysis

Previous experiments have shown that replacing the *pro*-R_P phosphoryl oxygen atom at position P9 in domain II of the conserved core and at the cleavage site (position 1.1) with a sulfur gave large deleterious rate effects in Mg^{2+} (Fig. 4). Furthermore, these deleterious thio effects (thio effect = $k_c^{wt}/k_c^{modified}$) could be substantially rescued upon addition of a thiophilic metal ion, such as Cd^{2+}, which has a higher affinity for sulfur than for oxygen (Knoll et al., 1997; Peracchi et al., 1997; Scott & Uhlenbeck, 1999; Wang et al., 1999; Feig, 2000). The simplest interpretation of these results is that the *pro*-R_P phosphoryl oxygen atoms at positions P9 and P1.1 are metal ion ligands in the divalent cation stimulated reaction.

If these atoms are indeed sites of divalent metal ion coordination, and if these sites are no longer used in the monovalent cation reaction, then the deleterious effect from sulfur substitution would be predicted to be greatly diminished in 4 M Li^+. As shown in Table 2, this prediction is indeed correct. The 10^3–10^4-fold thio effects observed in 10 mM Mg^{2+} are the same within error as those obtained previously for both HH16 and HH8 studies (Peracchi et al., 1997; Scott & Uhlenbeck, 1999; Wang et al., 1999), whereas the thio effects in 4 M Li^+ for both hammerheads at both positions are only ~10-fold.[8] As was observed previously in the presence of Mg^{2+} (Koizumi & Ohtsuka, 1991; Knoll et al., 1997; Scott & Uhlenbeck, 1999; Wang et al., 1999), there is also no significant thio effect in Li^+ from re-

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[7] A HH16 construct was used in this experiment having a 7-deazaguanine 2′-deoxyribose substitution at position 10.1. The 2′-deoxyribose substitution was previously shown to have no significant effect on catalysis (Wang et al., 1999).

[8] A somewhat larger thio effect of 50–70 is observed for the cleavage site sulfur substitution (P1.1) in 1 M Li^+, but not for the P9 substitution. This thio effect was unaffected by the presence or absence of added 0.1 mM Na_2EDTA, providing no indication that potentially contaminating metal ions are responsible. We do not understand the origin of this difference.

TABLE 2. Effect of sulfur substitutions at positions P9 and P1.1 on the hammerhead reaction in divalent and in monovalent cations[a]

		10 mM $MgCl_2$		4 M LiCl	
Parent ribozyme	Construct	k_c (min^{-1})	thio effect	k_c (min^{-1})	thio effect
HH16	O/N•O	0.42	NA	0.08	NA
	S_{R_P}/N•O	7.8×10^{-4}	550	1.6×10^{-2}	5
	O/N•S_{R_P}	1.4×10^{-4}	3,000	5.2×10^{-3}	15
HH8	O/N•O	1.1	NA	0.09	NA
	S_{R_P}/N•O	$\leq 1.7 \times 10^{-2}$[b]	≥ 65[b]	1.9×10^{-2}	5
	O/N•S_{R_P}	1.4×10^{-4}	7,900	7.4×10^{-3}	14

[a] All reactions were single turnover with a saturating excess of ribozyme relative to substrate and were carried out at 25 °C in 50 mM NaMOPS, pH 7.3 (see Methods). The rate constant k_c represents the reaction of the E•S complex. Reactions of HH16 and its variants were carried out with 200 nM ribozyme, and reactions of HH8 and its variants were carried out with 1 μM ribozyme. The same thio effects were obtained in other buffers. Errors were typically less than 30%. NA = not applicable.

[b] These values are limits because the observed rate constant could arise from a small amount of contaminating oxygen-containing ribozyme, as described previously (Peracchi et al., 1997).

placement of the *pro*-S_P phosphoryl oxygens at these positions (data not shown).

The substantial diminution of the *pro*-R_P thio effect in Li^+ is consistent with a role of these oxygens as divalent metal ion ligands that is absent in the monovalent cation reaction. The small remaining thio effect could be caused by conformational and electrostatic differences between normal phosphoryl groups and the sulfur-substituted ones, or by a small contribution of a monovalent cation binding at this position (Frey & Sammons, 1985; Smith & Nikonowicz, 2000; Suzumura et al., 2000).

Cd^{2+}-stimulation of reactions of thio-substituted hammerhead ribozyme in Li^+

To confirm that the divalent metal ion site involving P9 and P1.1 is not used in Li^+, rescue experiments were performed to determine whether the thiophilic divalent metal ion Cd^{2+} could stimulate catalysis of the phosphorothioate-substituted hammerheads in 4 M Li^+. When Cd^{2+} was added to hammerhead•substrate complexes containing an R_p-phosphorothioate at either P9 or P1.1 (Fig. 5, open squares and open circles, respectively), a 100-fold stimulation of the cleavage rate was observed. In contrast, no significant Mg^{2+}-stimulation was observed in the presence of 4 M Li^+. Conversely, for reactions of ribozyme•substrate complexes with phosphate or S_p-thiophosphate at P9 and P1.1 in the 4 M Li^+ reaction mix, addition of 10 mM $MgCl_2$ gave a 15-fold rate increase (data not shown), whereas addition of Cd^{2+} up to 100 mM had little effect (approximately threefold; data not shown).

Added Cd^{2+} shows a somewhat larger stimulation with wild-type HH16 when reactions are carried out in 10 mM Mg^{2+} or 10 mM Ca^{2+} (10- and 400-fold, respectively, with saturating Cd^{2+}) than in Li^+ (Fig. 5; Wang et al., 1999). The smaller stimulation afforded by Cd^{2+} observed in reaction mixes containing 4 M Li^+ may be due to a lower ability of Cd^{2+} to bind in high ionic strength and a lower concentration of free Cd^{2+} in the presence of high concentrations of Cl^- (Butler & Cogley, 1998). The Cd^{2+} effects described above and previously (Peracchi et al., 1997; Wang et al., 1999) show that the stimulation has the same stereochemis-

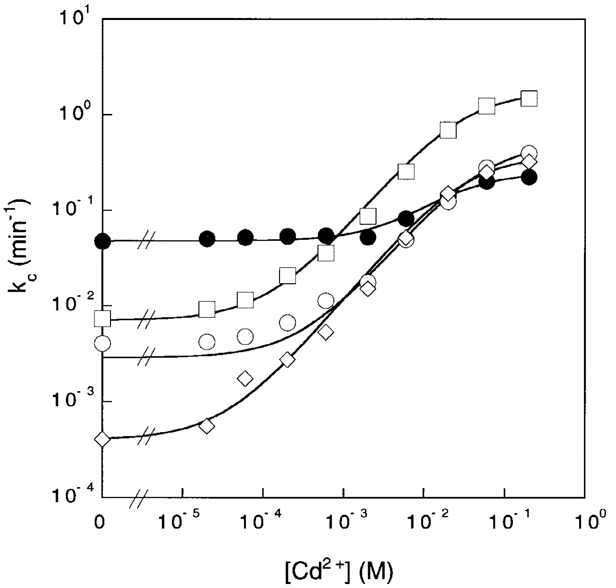

FIGURE 5. Cd^{2+} effect on hammerhead reactions in the presence of 4 M LiCl. Reactions of HH16 constructs O/N•O (●); O/N•S_{R_P} (○); S_{R_P}/N•O (□): S_{R_P}/N•S_{R_P} (◇) (see Fig. 4 for definitions of the construct). The data are fit to stimulation by a single Cd^{2+} ion, and give K_d^{app} and k_{max} of Cd^{2+} binding for O/N•O of 24 ± 4 mM and 0.20 ± 0.01 min^{-1}; O/N• S_{R_P} of 56 ± 5 mM and 0.5 ± 0.02 min^{-1}; S_{R_P}/N•O of 30 ± 3 mM and 1.7 ± 0.1 min^{-1}; S_{R_P}/N•S_{R_P} of 33 ± 2 mM and 0.38 ± 0.01 min^{-1}. Reaction conditions: 4 M LiCl, 20 μM Na_2EDTA, 50 mM BTP, pH 7.0, with varying concentrations of $CdCl_2$.

try with monovalent and divalent cations. Finally, the Cd^{2+} rescue is consistent with an effect from a single metal ion, even when both positions are simultaneously substituted with sulfur (Fig. 5, open diamonds), as was observed in Mg^{2+} or Ca^{2+} (Wang et al., 1999).

These results support the view that the sole substantial difference between hammerhead cleavage in monovalent and divalent cations is the stimulatory effect of a divalent metal ion bound to the P9/P1.1 site, an interaction that is absent or ineffectual under the high Li^+ conditions.

SUMMARY AND IMPLICATIONS

We have compared the cleavage activity of 24 modified hammerhead ribozymes in monovalent and divalent cations. Two major conclusions have emerged. The first is that the properties of hammerhead cleavage observed with monovalent cations are very similar to those of hammerhead cleavage in divalent cations. This conclusion was reached because nearly all of the modified hammerheads showed similar deleterious effects under both conditions, an occurrence that otherwise would be highly improbable. This finding is important because it provides strong evidence against the popular notion that much of the hammerhead's catalytic power derives from divalent metal ions acting to help deprotonate the attacking 2′-hydroxyl group or to stabilize the leaving 5′ oxygen atom. Although monovalent cations could potentially act in this way, albeit less effectively, the observation of considerable hammerhead catalysis in NH_4^+ and exchange-inert metal ions (Murray et al., 1998a; Curtis & Bartel, 2001) that cannot serve directly in the above-mentioned catalytic roles renders this unlikely. Thus, the most likely role for cations is simply to neutralize phosphate repulsion and facilitate the folding of the hammerhead core into a conformation that is active for cleavage.

The second major conclusion that emerged from this work is that a small part of the hammerhead rate enhancement does involve at least one divalent metal ion. This possibility was hinted at by the observation that the rate enhancement in monovalents is smaller than in divalents ($\sim10^4$ versus $\sim10^6$; Murray et al., 1998a; Curtis & Bartel, 2001; results herein). However, this difference could have reflected weaker binding of Li^+ or a structural defect. A smaller rate enhancement of 10^3–10^4 was also observed with exchange-inert Co(III) (Curtis & Bartel, 2001), again leaving open the possibility of a unique role for a divalent metal ion.

Two experiments presented herein suggest that a metal ion binding site, which involves the N7 of G10.1 and the *pro*-R_P phosphoryl oxygens of P9 and P1.1 and is important for catalysis in the presence of divalent metal ions, is not productively occupied in the presence of monovalent cations. First, when any of the three ligands at this site is disrupted, there is a large deleterious effect on the rate in Mg^{2+}, but not in Li^+. Second, the cleavage of a hammerhead ribozyme containing an R_P-phosphorothioate at either or both of the critical phosphoryl ligands is substantially enhanced in 4 M Li^+ upon the addition of Cd^{2+}, a thiophilic metal ion previously shown to function effectively at this site (Wang et al., 1999). Thus, the hammerhead appears unable to use its full complement of catalytic interactions unless this divalent metal site is occupied.

What, then, is the role of this divalent metal ion? It is possible that it has only a stabilizing role, to facilitate adoption of the catalytically active structure of the hammerhead by bridging the P9/P1.1 sites, which are ~20 Å apart in the X-ray structures (Wang et al., 1999). It is also possible that the metal ion interaction with a nonbridging phosphoryl oxygen atom at P1.1, the cleavage site, provides a modest amount of electrostatic stabilization to the reaction's transition state. Such electrostatic stabilization would be possible if there were an increase in negative charge on the nonbridging phosphoryl oxygens in going from the ground state to the transition state. This mechanism is commonly assumed, as analogous interactions are present in many protein enzymes. Nevertheless, nonenzymatic studies have not determined whether or not an increase in negative charge on the phosphoryl oxygens actually occurs. It should be recognized that it is extremely difficult to discern the mechanistic origin of rate enhancements even in model reactions catalyzed by metal ions (Cooperman, 1976; Thatcher & Kluger, 1989). Thus, it is difficult to distinguish a specific catalytic role in electrostatic stabilization of the transition state from other possible roles such as positioning the reactants or stabilizing the active conformation. It remains possible that the sole role of the P9/P1.1 metal ion interaction is to facilitate formation of the catalytic conformation of the hammerhead core.

In summary, the results of this and prior studies suggest that the primary role of divalent metal ions in hammerhead catalysis is to help the ribozyme fold into its active conformation. One divalent metal ion appears to interact directly with the cleavage site phosphoryl group and may provide a modest catalytic contribution from electrostatic stabilization of the transition state in addition to its role in facilitating formation of the active hammerhead structure. Nevertheless, most of the direct catalytic interactions do not involve metal ions. Uncovering the identity and role of the hammerhead functional groups that are involved in its catalysis presents an enormous and exciting future challenge.

MATERIALS AND METHODS

Materials

$CdCl_2$ (>99.99%), $MgCl_2$ (>99.99%) were purchased from Sigma-Aldrich. LiCl (>99%, with $Mg^{2+} < 5$ ppm) was Ultra-Sigma grade from Sigma-Aldrich. Buffers (>99%) were from Sigma or United States Biochemical. Recombinant T7 RNA polymerase was purified from an expression strain by standard procedures (Davanloo et al., 1984).

Wild-type and mutant HH8 ribozymes were synthesized by in vitro transcription using synthetic DNA templates and T7 RNA polymerase as previously described (Milligan et al., 1987). Wild-type and modified HH16 ribozymes and HH16 and HH8 substrate oligonucleotides were chemically synthesized by standard solid-phase phosphoramidite synthesis (Usman & Cedergren, 1992; Wincott et al., 1995; Baidya et al., 1997). RNAs were purified by polyacrylamide gel electrophoresis or HPLC prior to use. Stereoisomers of phosphorothioate-containing oligonucleotides were separated by anion-exchange HPLC and were assigned as described previously (Peracchi et al., 1997; Wang et al., 1999). Substrates for HH16 and HH8 ribozyme cleavage reactions were 5′-end labeled with [γ^{32}P]-ATP and T4 polynucleotide kinase and purified by gel electrophoresis prior to use.

The sequences and numbering for the HH16 and HH8 ribozyme and substrates are shown in Figure 1A and 1B, respectively. The structure and nomenclature for phosphorothioate-containing ribozymes and substrates are shown in Figure 4.

Methods

Kinetic experiments were carried out under single-turnover conditions by using a large excess of ribozyme (50–300 nM for HH16 and 1.0 μM for HH8) and trace quantities of radiolabeled substrate ($<$0.1 nM). To prevent affects from contaminating metals, 0.1 mM Na_2EDTA was present in the reactions unless stated otherwise. Addition of 40 mM sodium diethyldithiocarbamate gave no observable precipitate, nor did this addition or addition of 100 mM EDTA affect the observed rate constants, suggesting that there was no significant divalent metal ion contamination (Derrick et al., 2000). Unless otherwise noted, the ribozyme and substrate oligonucleotides were combined in the appropriate buffer for a given experiment in the absence of the added monovalent or divalent ions, incubated for 1–2 min at 95 °C, and then equilibrated at 25 °C for 5–20 min to allow proper annealing. A slightly altered annealing condition was used for the HH8 reactions containing phosphorothioate substitutions and corresponding controls, in which the ribozyme and substrate oligonucleotides were incubated in separate tubes at 95 °C for 2 min, then combined at 25 °C. Reactions were initiated by mixing the E•S complex with the appropriate divalent or monovalent ion solution to give the desired final concentrations. Aliquots of the kinetic reactions were quenched at the appropriate times by dilution into a solution containing 85% formamide or 8 M urea, a molar excess of EDTA over any divalent metal ion present, and dyes (bromophenol blue and xylene cyanol). Controls indicated that the quench was effective for all reaction conditions. The cleavage products were separated from unreacted substrate by electrophoresis in 20% polyacrylamide/7 M urea gels. Radioactivity was quantitated by using a Molecular Dynamics PhosphorImager. Rate constants (k_c) were obtained from plots of product formed versus time (KaleidaGraph; Synergy Software). Unless noted otherwise, the data fit well to single exponential curves with endpoints of ~80% as previously observed (Hertel et al., 1994, 1998; Stage-Zimmermann & Uhlenbeck, 1998; Wang et al., 1999). Reactions with phosphorothioate-containing HH16 ribozymes or with HH16 or HH8 phosphorothioate-containing substrates were fit to a double exponential for two independent exponential processes to account for small levels of contamination by the other thio-isomer and RNA lacking the sulfur substitution, as described previously (Peracchi et al., 1997; Wang et al., 1999). The kinetic phases are readily separable, and the rate constants obtained are highly reproducible and reliable. The slowest reactions were followed for 1–3 days and k_c was obtained from the early portion of the reaction progress curve assuming an endpoint of 80%.

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REFERENCES

Baidya N, Ammons GE, Matulic-Adamic J, Karpeisky AM, Beigelman L, Uhlenbeck OC. 1997. Functional groups on the cleavage site pyrimidine nucleotides are required for stabilization of the hammerhead transition state. *RNA 3*:1135–1142.

Baidya N, Uhlenbeck OC. 1997. A kinetic and thermodynamic analysis of cleavage site mutations in the hammerhead ribozyme. *Biochemistry 36*:1108–1114.

Ban N, Nissen P, Hansan J, Moore PB, Steitz, TA. 2000. The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution. *Science 289*:905–920.

Butler J, Cogley D. 1998. *Ionic Equilibrium: Solubility and pH Calculations*. New York: John Wiley and Sons.

Clouet-d'Orval B, Uhlenbeck OC. 1997. Hammerhead ribozymes with a faster cleavage rate. *Biochemistry 36*:9087–9092.

Cooperman BS. 1976. The role of divalent metal ions in phosphoryl and nucleotidyl transfer. In: Sigel H, ed. *Metal ions in biological systems*, Vol. 5. New York: Marcel Dekker. pp 79–125.

Curtis E, Bartel DP. 2001. The hammerhead cleavage reaction in monovalent cations. *RNA.* In this issue.

Dahm SC, Derrick WB, Uhlenbeck OC. 1993. Evidence for the role of solvated metal hydroxide in the hammerhead cleavage mechanism. *Biochemistry 32*:13040–13045.

Dahm SC, Uhlenbeck OC. 1991. Role of divalent metal ions in the hammerhead RNA cleavage reaction. *Biochemistry 30*:9464–9469.

Davanloo P, Rosenberg AH, Dunn JJ, Studier FW. 1984. Recombinant T7 RNA polymerase was purified from an expression strain by standard procedures. *Proc Natl Acad Sci USA 81*:2035–2039.

Derrick WB, Greef CH, Caruthers MH, Uhlenbeck OC. 2000. Hammerhead cleavage of the phosphorodithioate linkage. *Biochemistry 39*:4947–4954.

Draper DE. 1996. Strategies for RNA folding. *Trends Biochem Sci 21*:145–149.

Fedor MJ. 2000. Structure and function of the hairpin ribozyme. *J Mol Biol 297*:269–291.

Fedor MJ, Uhlenbeck OC. 1992. Kinetics of intermolecular cleavage by hammerhead ribozymes. *Biochemistry 31*:12042–12054.

Feig AL, Uhlenbeck OC. 1999. The role of metal ions in RNA biochemistry. In: Gesteland RF, Cech TR, Atkins JF, eds. *The RNA world*, 2nd ed., Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 287–319.

Feig AL. 2000. The use of manganese as a probe for elucidating the role of magnesium ions in ribozymes. In: Sigel H, Sigel A, eds.

Metal ions in biological systems 37. New York: Marcel Dekker. pp 157–182.

Ferre-D'Amare AR, Zhou K, Doudna JA. 1998. Crystal structure of a hepatitis delta virus ribozyme. *Nature 395*:567–574.

Forster AC, Symons RH. 1987a. Self-cleavage of plus and minus RNAs of a virusoid and a structural model for the active sites. *Cell 49*:211–220.

Forster AC, Symons RH. 1987b. Self-cleavage of virusoid RNA is performed by the proposed 55-nucleotide active site. *Cell 50*:9–16.

Frey PA, Sammons RD. 1985. Bond order and charge localization in nucleoside phosphorothioates. *Science 228*:541–545.

Geyer CR, Sen D. 1997. Evidence for the metal-cofactor independence of an RNA phosphodiester-cleaving DNA enzyme. *Chem Biol 4*:579–593.

Hampel A, Cowan JA. 1997. A unique mechanism for RNA catalysis: The role of the metal cofactors in hairpin ribozyme cleavage. *Chem Biol 4*:513–517.

Hertel KJ, Herschlag D, Uhlenbeck OC. 1994. A kinetic and thermodynamic framework for the hammerhead ribozyme reaction. *Biochemistry 33*:3374–3385.

Hertel KJ, Pardi A, Uhlenbeck OC, Koizumi M, Ohtsuka E, Uesugi S, Cedergren R, Eckstein F, Gerlach WL, Hodgson R. 1992. Numbering system for the hammerhead. *Nucleic Acids Res 20*:3252.

Hertel K, Stage-Zimmermann TK, Ammons G, Uhlenbeck OC. 1998. Thermodynamic dissection of the substrate-ribozyme interaction in the hammerhead ribozyme. *Biochemistry 37*:16983–16988.

Jayasena VK, Gold L. 1997. In vitro selection of self-cleaving RNAs with a low pH optimum. *Proc Natl Acad Sci USA 94*:10612–10617.

Knoll R, Bald R, Furste JP. 1997. Complete identification of nonbridging phosphate oxygens involved in hammerhead cleavage. *RNA 3*:132–140.

Koizumi M, Ohtsuka E. 1991. Effects of phosphorothioate and 2-amino groups in hammerhead ribozymes on cleavage rates and Mg^{2+} binding. *Biochemistry 30*:5145–5150.

Lott WB, Pontius BW, Von Hippel PH. 1998. A two-metal ion mechanism operates in the hammerhead ribozyme-mediated cleavage of an RNA substrate. *Proc Natl Acad Sci USA 95*:542–547.

McKay DB. 1996. Structure and function of the hammerhead ribozyme: An unfinished story. *RNA 2*:395–403.

Milligan JF, Groebe DR, Witherell GW, Uhlenbeck OC. 1987. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acids Res 15*:8783–8798.

Murray JB, Seyhan AA, Walter NG, Burke JM, Scott WG. 1998a. The hammerhead, hairpin and VS ribozymes are catalytically proficient in monovalent cations alone. *Chem Biol 5*:587–595.

Murray JB, Szoke H, Szoke A, Scott WG. 2000. Capture and visualization of a catalytic RNA enzyme–product complex using crystal lattice trapping and X-ray holographic reconstruction. *Mol Cell 5*:279–287.

Murray JB, Terwey DP, Maloney L, Karpeisky A, Usman N, Beigelman L, Scott WG. 1998b. The structural basis of hammerhead ribozyme self-cleavage. *Cell 92*:665–673.

Nakano S, Chadalavada DM, Bevilacqua PC. 2000. General acid-base catalysis in the mechanism of a hepatitis delta virus ribozyme. *Science 27*:1493–1497.

Narlikar GJ, Herschlag D. 1997. Mechanistic aspects of enzymatic catalysis: Lessons from comparison of RNA and protein enzymes. *Annu Rev Biochem 66*:19–59.

Nesbitt S, Hegg LA, Fedor MJ. 1997. An unusual pH-independent and metal-ion-independent mechanism for hairpin ribozyme catalysis. *Chem Biol 4*:619–630.

Nissen P, Hansen J, Ban N, Moore PB, Steitz TA. 2000. The structural basis of ribosome activity in peptide bond synthesis. *Science 289*:920–930.

Pan T, Long DM, Uhlenbeck OC. 1993. Divalent metal ions in RNA folding and catalysis. In: Gesteland RF, Atkins JF, eds. *The RNA world*, 1st ed., Plainview, New York: Cold Spring Harbor Laboratory Press. pp 271–301.

Peracchi A, Beigelman L, Scott EC, Uhlenbeck OC, Herschlag D. 1997. Involvement of a specific metal ion in the transition of the hammerhead ribozyme to its catalytic conformation. *J Biol Chem 272*:26822–26826.

Peracchi A, Beigelman L, Usman N, Herschlag D. 1996. Rescue of abasic hammerhead ribozymes by exogenous addition of specific bases. *Proc Natl Acad Sci USA 93*:11522–11527.

Peracchi A, Karpeisky A, Maloney L, Beigelman L, Herschlag D. 1998. A core folding model for catalysis by the hammerhead ribozyme accounts for its extraordinary sensitivity to abasic mutations. *Biochemistry 37*:14765–14775.

Perreault JP, Labuda D, Usman N, Yang JH, Cedergren R. 1991. Relationship between 2′-hydroxyls and magnesium binding in the hammerhead RNA domain: A model for ribozyme catalysis. *Biochemistry 30*:4020–4025.

Perrotta AT, Shih I, Been MD. 1999. Imidazole rescue of a cytosine mutation in a self-cleaving ribozyme. *Science 286*:123–126.

Pley HW, Flaherty KM, McKay DB. 1994. Three-dimensional structure of a hammerhead ribozyme. *Nature 372*:68–74.

Pontius BW, Lott WB, Von Hippel PH. 1997. Observations on catalysis by hammerhead ribozymes are consistent with a two-divalent-metal ion mechanism. *Proc Natl Acad Sci USA 94*:2290–2294.

Pyle AM. 1996. Role of metal ions in ribozymes. In: Sigel H, ed. *Metal ions in biological systems 32*. New York: Marcel Dekker. pp 479–520.

Roth A, Breaker RB. 1998. An amino acid as a cofactor for a catalytic polynucleotide. *Proc Natl Acad Sci USA 95*:6027–6031.

Scott EC, Uhlenbeck OC. 1999. A re-investigation of the thio effect at the hammerhead cleavage site. *Nucleic Acids Res 27*:479–484.

Scott WG, Finch JT, Klug A. 1995. The crystal structure of an all-RNA hammerhead ribozyme: A proposed mechanism for RNA catalytic cleavage. *Cell 81*:991–1002.

Scott WG, Klug A. 1996. Ribozymes: Structure and mechanism in RNA catalysis. *Trends Biochem Sci 21*:220–224.

Scott WG, Murray JB, Arnold JRP, Stoddard BL, Klug A. 1996. Capturing the structure of a catalytic RNA intermediate: The hammerhead ribozyme. *Science 274*:2065–2069.

Slim G, Gait MJ. 1991. Configurationally defined phosphorothioate-containing oligoribonucleotides in the study of the mechanism of cleavage of hammerhead ribozymes. *Nucleic Acids Res 19*:1183–1188.

Smith JS, Nikonowicz EP. 2000. Phosphorothioate substitution can substantially alter RNA conformation. *Biochemistry 39*:5642–5652.

Stage-Zimmermann TK, Uhlenbeck OC. 1998. Hammerhead ribozyme kinetics. *RNA 4*:875–889.

Steitz TA, Steitz JA. 1993. General two-metal-ion mechanism for catalytic RNA. *Proc Natl Acad Sci USA 90*:6498–6502.

Suga H, Cowan JA, Szostak JW. 1998. Unusual metal ion catalysis in an acyl-transferase ribozyme. *Biochemistry 37*:10118–10125.

Suzumura K, Warashina M, Yoshinari K, Tanaka Y, Kuwabara T, Orita M, Taira K. 2000. Significant change in the structure of a ribozyme upon introduction of a phosphorothioate linkage at P9: NMR reveals a conformational fluctuation in the core region of a hammerhead ribozyme. *FEBS Lett 473*:106–112.

Taira K, Uebayasi M, Maeda H, Furukawa K. 1990. Energetics of RNA cleavage: Implications for the mechanism of action of ribozymes. *Prot Eng 3*:691–701.

Thatcher GRJ, Kluger R. 1989. Mechanism and catalysis of nucleophilic substitution in phosphate esters. *Adv Phy Org Chem 25*:99–265.

Uhlenbeck OC. 1987. A small catalytic oligoribonucleotide. *Nature 328*:596–600.

Usman N, Cedergren R. 1992. Exploiting the chemical synthesis of RNA. *Trends Biochem Sci 17*:334–339.

Vaish NK, Kore AR, Eckstein F. 1998. Recent developments in the hammerhead ribozyme field. *Nucleic Acids Res 26*:5237–5242.

Wang S, Karbstein K, Peracchi A, Beigelman L, Herschlag D. 1999. Identification of the hammerhead ribozyme metal ion binding site responsible for rescue of the deleterious effect of a cleavage site phosphorothioate. *Biochemistry 38*:14363–14378.

Wincott F, DiRenzo A, Shaffer C, Grimm S, Tracz D, Workman C, Sweedler D, Gonzalez C, Scaringe S, Usman N. 1995. Synthesis, deprotection, analysis and purification of RNA and ribozymes. *Nucleic Acids Res 23*:2677–2684.

Yarus M. 1993. How many catalytic RNAs? Ions and the Cheshire cat conjecture. *FASEB J 7*:31–39.

Yoshinari K, Taira K. 2000. A further investigation and reappraisal of the thio effect in the cleavage reaction catalyzed by a hammerhead ribozyme. *Nucleic Acids Res 28*:1730–1742.

Young KJ, Gill F, Grasby JA. 1997. Metal ions play a passive role in the hairpin ribozyme catalyzed reaction. *Nucleic Acids Res 25*:3760–3766.

Zhou DM, Taira K. 1998. The hydrolysis of RNA: From theoretical calculations to the hammerhead ribozyme-mediated cleavage of RNA. *Chem Rev 98*:991–1026.