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LETTER TO THE EDITOR

Relating conformation, Mg²⁺ binding, and functional group modification in the hammerhead ribozyme

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INTRODUCTION

The study of structure–function relationships in RNAs have traditionally involved the evaluation of functional change incurred by chemical modifications or site-specific replacements of one nucleotide by another. The development of RNA chemical synthesis provided an alternate strategy where changes in RNA behavior were observed as a result of the incorporation of modified nucleotides containing deletions or modifications of specific functional groups of the base, sugar, or phosphate moieties (Usman & Cedergren, 1992). This use of “functional group modifications” or mutagenesis (FGM) in structure–function relationships has the perceived advantage that only slight conformational perturbations might be expected in the modified RNA, rather than the more drastic changes anticipated from nucleotide replacement. How well FGM actually performs in probing an RNA structure is, however, somewhat controversial. This controversy extends as well to other methods of FGM, such as chemical modification. The availability of X-ray crystal structures of the “ground state” hammerhead domain (Pley et al., 1994; Scott et al., 1995; Fig. 1) could have provided a firm basis for interpreting the FGM data, but only served to deepen the controversy, because major discrepancies have been pointed out (Tuschl et al., 1995; McKay, 1996; Usman et al., 1996). It is thus interesting that the metal binding and configurational intermediate of the hammerhead structure published recently (Scott et al., 1996) help explain some of the apparent discrepancies between the FGM and crystallographic data.

FGM AND MAGNESIUM ION BINDING SITES

The most critical functional groups detected by FGM experiments are shown in Table 1 (for a complete list

of FGM positions see Tuschl et al., 1995; McKay, 1996; Usman et al., 1996). The correlation of these data with the functional groups implicated in magnesium binding sites is very striking: among the 15 groups listed, 10 are involved in magnesium coordination. In fact, most of the functional groups involved in the formation of sites 2, 3, 4, and 6 (Scott et al., 1996) have been detected by FGM analysis. Among the five other FGM hits, one (the 2' OH of U_{16.1}) is involved in hydrogen bonding and the other four (the N7 of A₆, the O1P of A₁₃ and A₁₄ as well as the N3 of A₁₃) do not seem to interact with other atoms in the three-dimensional structures (Pley et al., 1994; Scott et al., 1995, 1996).

This high correlation and the importance of these sites prompted us to examine the possibility of predicting metal ion binding pockets in a given RNA structure. Because these sites should be electronegative to accommodate cations, one may be able to identify them by determining the electronegative potential landscape of the three-dimensional structures. This technique has been used to predict metal ion binding sites in proteins (Weiss et al., 1991; Karshikoff et al., 1994) and to analyze these sites in RNA (Cate & Doudna, 1996). To demonstrate the utility of this computation, the electronegative landscape for the three-dimensional structure of yeast tRNA^{Phe} has been calculated (Westhof & Sundaralingam, 1986), and the five negatively charged regions that were found (Fig. 2A) correspond to the known strong magnesium binding sites (Holbrook et al., 1977). Next, we repeated this computation on the published crystal structures of the hammerhead ribozyme (Fig. 2B,C). Scott et al. (1995) had placed five magnesium ions in the crystal structure, whereas Pley et al. (1994) gave the location of a single cation binding site. The more recent work of Scott et al. (1996) also shows five cation binding sites but includes two new ones, labeled sites 4 and 6 (the original site 4 is no longer present).

The magnesium ion binding sites 1–4 shown in the crystal structure of Scott et al. (1995) were detected

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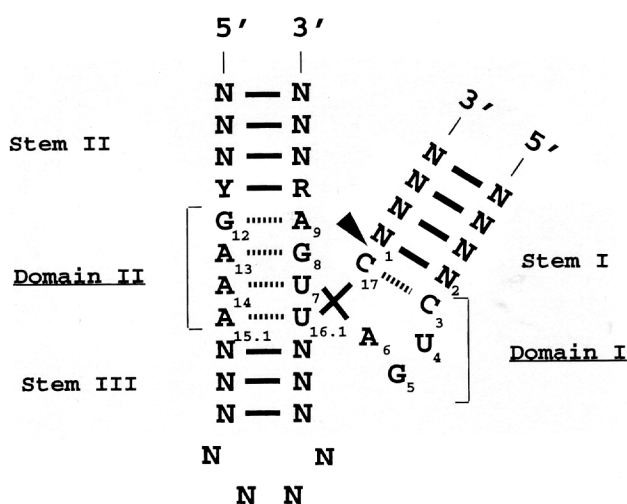


FIGURE 1. Secondary structure of the hammerhead reflecting the tertiary structure. The cleavage site is indicated by an arrow.

readily in the electronegative potential distribution (Fig. 2B,C). We could find these same sites in the structure of Pley et al. (1994). On the other hand, the electronegative potential criterion does not support site 5 (Scott et al., 1995). Interestingly, site 1 is found in both the Scott et al. (1995) and the Pley et al. (1994) structure, even though it is formed by different nucleotides in positions 2.2 and 2.3 (G, G in Scott and C, A in Pley). This observation may demonstrate the three-dimensional degeneracy of RNA primary sequences.

The different states of the hammerhead ribozyme-substrate complex published recently by Scott et al. (1996) consist of a "ground state" structure isolated at low pH and an apparent configurational intermediate

accumulating prior to cleavage at high pH. Comparison of the electronegative landscapes and Mg^{2+} binding sites of these two structures reveals that some binding sites are pH sensitive and can shift as much as 6.8 Å; that is, site 1 is shifted 1.4 Å, site 2 is shifted 6.8 Å, and site 3 is shifted 2.3 Å (Fig. 2B). Furthermore, the additional metal ion binding site (site 6 in Scott et al., 1996) identified as catalytic is present in a moderately electronegative region composed of nucleotides $A_{1.1}$ and C_{17} (Fig. 2C). However, the proximity (4.46 Å) of sites 1 and 6 suggests that they share a water molecule in their inner coordination sphere, possibly rendering them more dependent on geometric constraints in coordinating backbone phosphates than on electrostatic properties of this region. In contrast, the region around nucleotides A_{13} , A_{14} , and $A_{15.1}$ is highly electronegative, but is unoccupied in these structures (Fig. 2D). This negatively charged pocket is also present, but unoccupied, in the structures published by Pley et al. (1994) and Scott et al. (1995).

In considering the electronegativity data, we recalled thiophosphate-replacement experiments at the O1P of nucleotides A_{13} and A_{14} suggesting that these phosphates could be involved in the binding of a magnesium cation (Ruffner & Uhlenbeck, 1990; Uhlenbeck, 1995; Knoll et al., 1997). This potential magnesium binding pocket formed additionally by the N1 of G_8 (Fig. 3) is consistent with the reduced activity of a hammerhead analogue containing a modification at the N1 at G_8 obtained via an O6-methyl guanosine and its increased K_{mg} (Grasby et al., 1993). Moreover, the presence of a 1-methylguanosine at position 8 also decreases the activity of this ribozyme (Limauro et al., 1994), and a magnesium binding site in this region would explain the magnesium ion-mediated changes in the fluorescence of the 2-aminopurine analogue at position 7 (Menger et al., 1996). The absence of magnesium ion in this region could be explained by its inaccessibility to $Mg(H_2O)_6^{2+}$, the dominant binding species. However, we do not rule out the possibility that this site could exist as a transient in the folding pathway of the hammerhead or in the transition-state structure. Indeed, NMR data of the cleaved ribozyme suggests that the region of U_7 is quite flexible, raising the possibility that modifying its interaction with A_{14} could provoke sufficient conformational change to open an otherwise closed binding site.

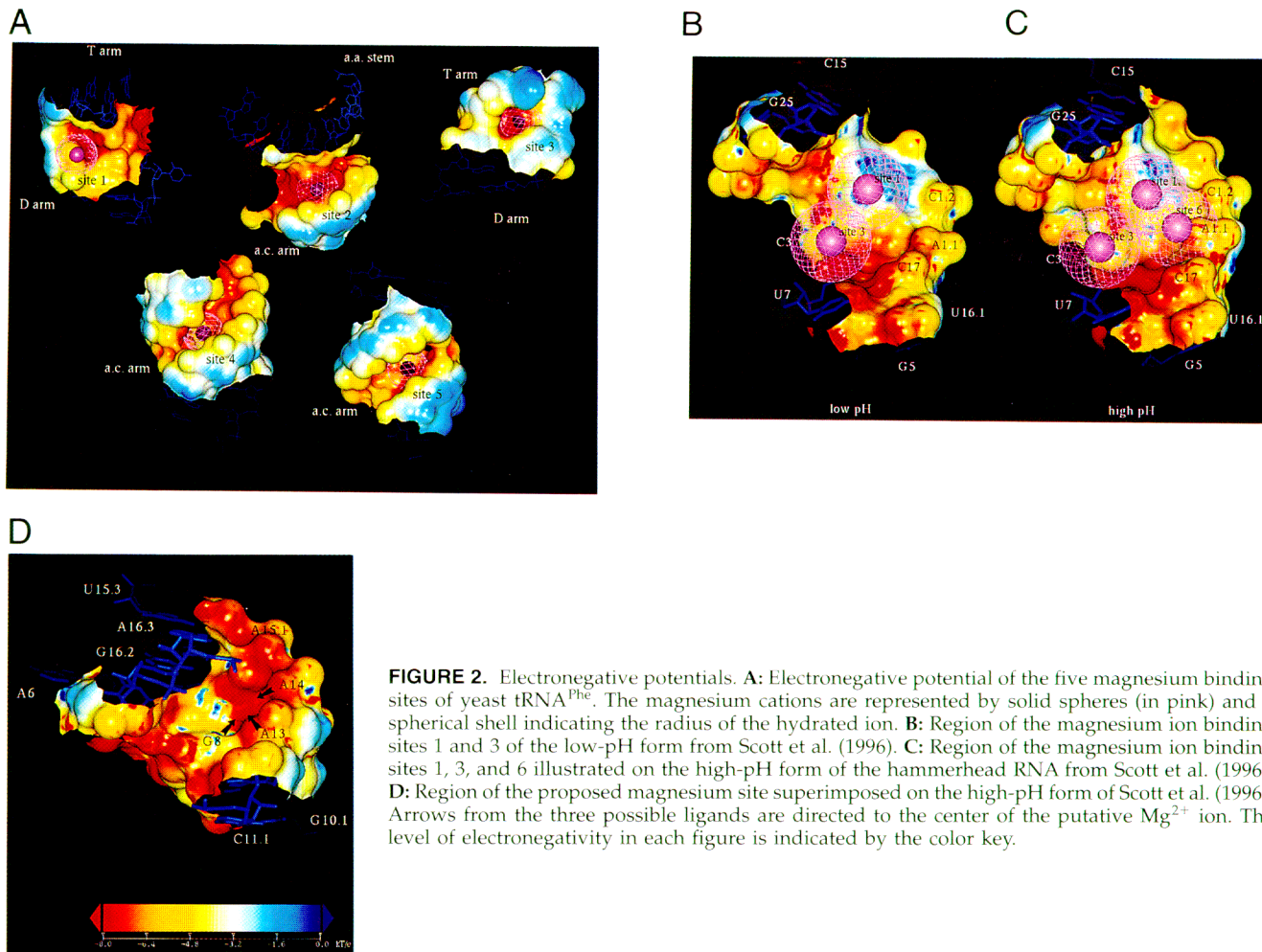
TABLE 1. Functional groups giving greater than a 10× effect on activity when modified.^a

Position	Functional group altered	Decrease in activity
$N_{1.1}$	<u>O1P</u>	$\gg 10\times$
C_3	<u>N4</u>	$>10\times$
G_5	<u>2'-OH</u>	$\gg 10\times$
	<u>N2</u>	$\gg 10\times$
A_6	<u>N7</u>	$>10\times$
G_8	<u>2'-OH</u>	$\gg 10\times$
A_9	<u>O1P</u>	$<10\times$
G_{12}	<u>N2</u>	$\gg 10\times$
A_{13}	<u>N3</u>	$10\times$
	<u>O1P</u>	$>10\times$
A_{14}	<u>O1P</u>	$>10\times$
$A_{15.1}$	<u>N3</u>	$>10\times$
$U_{16.1}$	<u>2'-OH</u>	$>10\times$
C_{17}	<u>2'-OH</u>	No cleavage
	<u>N4</u>	$10\times$

^aFunctional groups involved in magnesium ion binding are underlined (adapted from McKay, 1996).

FGM AND BASE PAIRING INTERACTIONS

In contrast to magnesium binding sites, base pairing interactions are proving much more difficult to detect using FGM. Examples of locations where FGM gives only modest effects are shown in Table 2. Those underlined are located within the highly conserved Domain II (positions 7-9 and 12-16.1, Fig. 1) and are involved in the hydrogen bonds of the non-Watson-



Crick interactions of these nucleotides. In an effort to rationalize discrepancies between FGM data and X-ray structures, McKay (1996) pointed to the possibility that these core nucleotides (Fig. 1) could participate in low-energy hydrogen bonding (Turner & Bevilacqua, 1993). This type of interaction has been observed in the GAAA tetraloop region by FGM analysis. It may be more than coincidence that the low-energy hydrogen bonds in the tetraloop are also involved in non-Watson-Crick interactions (SantaLucia et al., 1992). However, what actually constitutes a low-energy hydrogen bond and its physical basis remains somewhat obscure at this point in time.

Alternatively, folding could provide a rationale for the FGM data. The hammerhead core has been shown to undergo several major steps in folding, even after binding of the substrate, in order to reach the ground-state conformation represented by the crystal structures (Bassi et al., 1995, 1996). If these folding steps were slow enough, a hydrogen bond made or broken during a conformational change could affect the observed activity of the hammerhead even if it were not formally present in either the ground or the transition-state structure. However, conformational changes of

RNA, for example those involved during the formation of base pairs, are generally considered to be rapid and certainly much faster than the chemical step of catalysis. In fact, rates for some steps in the folding pathway of both the Group I intron core and RNase P are on the order of 0.7 min⁻¹ (Zarrinkar et al., 1994, 1996), incredibly close to the reaction rate of many RNA-catalyzed reactions, including that of the hammerhead. It is tempting to speculate that the similarity

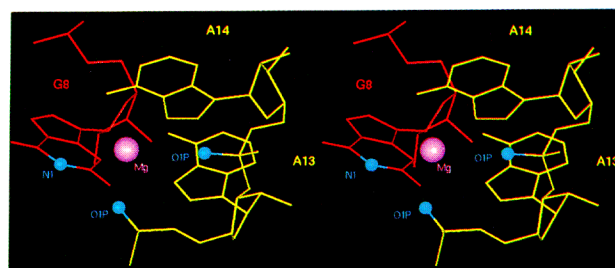


FIGURE 3. Stereo structure of the proposed magnesium binding site. Functional groups involved in magnesium ion binding are indicated by blue spheres and correspond to the N1 of G₈ and the O1P of A₁₃ and A₁₄. Pink sphere represents the magnesium cation.

TABLE 2. Functional groups having a less than 10× effect on activity when modified.^a

Position	Functional group altered	Decrease in activity
N _{1.1}	O2P	None
U ₄	2'-OH	<10×
	O4	<10×
G ₅	N7	<10×
A ₆	2'-OH	<10×
	N3	<10×
	N6	<10×
U ₇	2'-OH	None
G ₈	<u>N2</u>	1-12×
	N7	<10×
A ₉	2'-OH	<10×
	N3	<10×
	<u>N6</u>	1-6×
	<u>N7</u>	2-10×
G ₁₂	2'-OH	None
	N7	<10×
A ₁₃	2'-OH	<10×
	<u>N6</u>	7-17×
	<u>N7</u>	2-4×
A ₁₄	2'-OH	<10×
	N3	<10×
	<u>N6</u>	3-12×
	<u>N7</u>	3-14×
A _{15.1}	2'-OH	<10×
	<u>N6</u>	<10×
	<u>N7</u>	<10×
U _{16.1}	<u>O4</u>	1.5×

^aFunctional groups involved in base pairing interactions are underlined (adapted from McKay, 1996).

of catalytic rates for ribozymes could depend at least partially on conformational changes.

The fact that RNA folds in steps via identifiable intermediates and that some of these steps require Mg²⁺ (Zarrinkar et al., 1994, 1996; Menger et al., 1996) suggests that favored pathways would be those that provide the proper scaffolding for the formation of Mg²⁺ pockets. Indeed, Bassi et al. (1996) have shown that the formation of the purine-purine base pairs of the Domain II during the folding of the hammerhead RNA requires and is thermodynamically driven by the binding of magnesium cations to the ribozyme. Base pairing interactions in this domain may, therefore, fulfill a geometric rather than a thermodynamic requirement of the reaction. Modifications in the Domain II region could affect the folding pathway by changing the geometry between nucleotides, thereby reducing the affinity of the cation for the binding pocket (Bassi et al., 1996).

In support of this hypothesis, FGM in the Domain II generally involves deletion of a single functional group from a purine; thus, no major change in the geometry of this purine-rich region with respect to that of the wild-type molecule would be expected. Therefore, the incorporation of the "deleted"-type nucleotide ana-

logues in this region should result only in minor decreases of activity. On the other hand, the more dramatic effect on activity of using nucleotides modified in more than one functional group such as isoguanosine, 2-aminopurine, or xanthosine (Tuschl et al., 1993), as well as nucleotide replacement could be rationalized by their greater interference with the folding pathway both in terms of juxtapositions of nucleotides and interactions with cations. This explanation might deal with the apparent paradox that, although FGM in this region does not have drastic effects on activity, nucleotide replacements in Domain II render the molecule inactive.

METHODS

The X-ray crystallographic structures of the hammerhead ribozyme (#1HMH and #1MME) were obtained from the Protein Data Bank at the Brookhaven National Library. After addition of protons, the structures were refined by energy minimization until the maximum derivative was less than 50 kcal/Å, using the Discover software package (Discover 2.97, Molecular Simulations Inc., San Diego 1995) interfaced to the AMBER force field.

The electrostatic potentials were calculated using the finite difference nonlinear Poisson-Boltzmann equation (Nicholls & Honig, 1991) implemented in the program Delphi (Delphi 95.0, Molecular Simulations Inc., San Diego 1995) and the results displayed with Insight II. Details of the finite difference procedure to calculate the electrostatic potentials are described elsewhere (Misra et al., 1994). The solid accessible surface of the hammerhead RNA molecule was described by rolling a spherical probe corresponding to the radius of a water molecule (1.4 Å), around the exterior of the molecule. The molecules were assumed to be a low dielectric medium enclosed within the volume described by the surface. The partial charge distribution was taken from the amber force field parameters.

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