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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

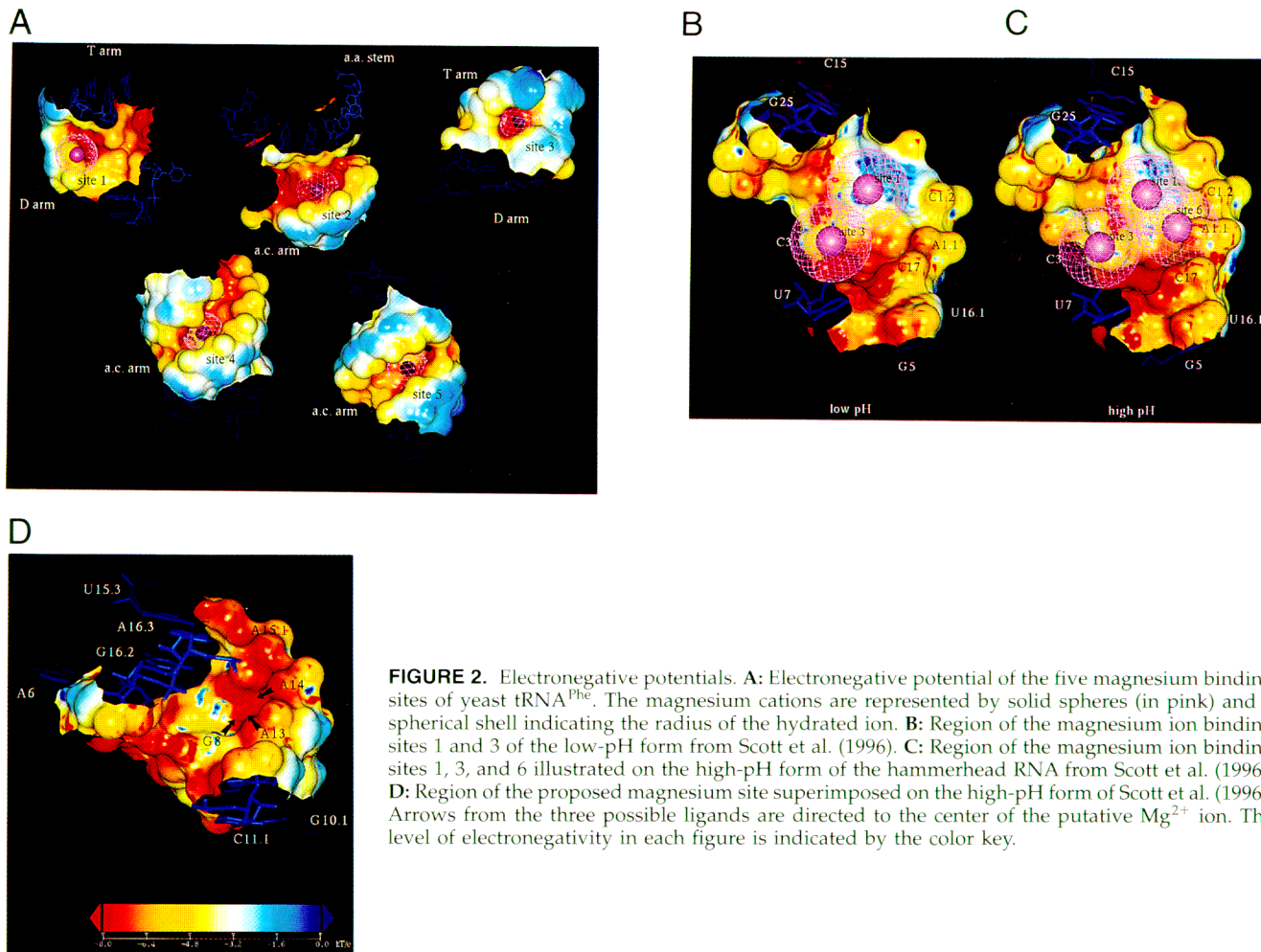
The most critical functional groups detected by FGM experiments are shown in Table 1 (for a complete list

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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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

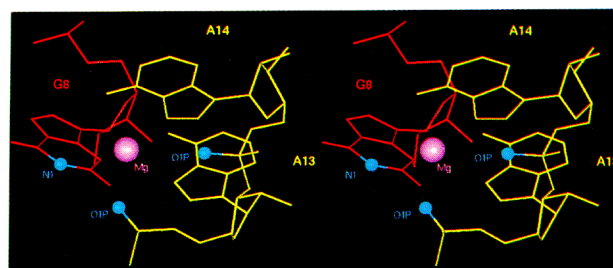


FIGURE 3. Stereo structure of the proposed magnesium binding site. Functional groups involved in magnesium ion binding are indicated by blue spheres and correspond to the N1 of G₈ and the O1P of A₁₃ and A₁₄. Pink sphere represents the magnesium cation.

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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