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Exploring purine N7 interactions via atomic mutagenesis: The group I ribozyme as a case study

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ABSTRACT

Atomic mutagenesis has emerged as a powerful tool to unravel specific interactions in complex RNA molecules. An early extensive study of analogs of the exogenous guanosine nucleophile in group I intron self-splicing by Bass and Cech demonstrated structure–function relationships analogous to those seen for protein ligands and provided strong evidence for a well-formed substrate binding site made of RNA. Subsequent functional and structural studies have confirmed these interacting sites and extended our understanding of them, with one notable exception. Whereas 7-methyl guanosine did not affect reactivity in the original study, a subsequent study revealed a deleterious effect of the seemingly more conservative 7-deaza substitution. Here we investigate this paradox, studying these and other analogs with the more thoroughly characterized ribozyme derived from the *Tetrahymena* group I intron. We found that the 7-deaza substitution lowers binding by ~20-fold, relative to the cognate exogenous guanosine nucleophile, whereas binding and reaction with 7-methyl and 8-aza-7-deaza substitutions have no effect. These and additional results suggest that there is no functionally important contact between the N7 atom of the exogenous guanosine and the ribozyme. Rather, they are consistent with indirect effects introduced by the N7 substitution on stacking interactions and/or solvation that are important for binding. The set of analogs used herein should be valuable in deciphering nucleic acid interactions and how they change through reaction cycles for other RNAs and RNA/protein complexes.

Keywords: group I ribozyme; atomic mutagenesis; purine N7

INTRODUCTION

Atomic mutagenesis is a powerful tool in which individual atoms or functional groups are replaced by atoms or groups with different properties. Such substitutions are readily made in nucleosides and incorporated into RNA molecules. This ability, coupled with an initial dearth of structural information, led to extensive early structure–function studies of RNAs (see Waring 1989; Christian and Yarus 1992; Sontheimer et al. 1997; Strobel and Shetty 1997; Blount and Uhlenbeck 2005; Das and Piccirilli 2005; Hougland et al. 2005; Soukup and Soukup 2009; Chirkova et al. 2010, and references therein). The power of these approaches to identify atomic-level interactions has been confirmed by subsequent X-ray structural models, and the functional studies have at times resolved uncertainties from structural models and, more generally, have greatly extended our understanding of the energetic and functional properties of these RNAs (e.g., Blount and Uhlenbeck 2005; Hougland et al. 2006; Forconi et al. 2009).

A classic in RNA atomic mutagenesis is Bass and Cech's 1984 use of a battery of guanosine analogs to map out interactions with the *Tetrahymena* group I intron, which uses an exogenous guanosine molecule (G) as a nucleophile to initiate self-splicing (Bass and Cech 1984). Their results implicated a subset of atoms important for guanosine binding and reactivity that was largely confirmed by subsequent structural (Adams et al. 2004; Golden et al. 2005; Guo et al. 2005; Stahley and Strobel 2005) and functional data (Michel et al. 1989; Sjogren et al. 1997; Shan and Herschlag 1999; Shan et al. 1999; Forconi et al. 2009, 2011). However, one uncertainty has remained—regarding the role of the N7 atom of G.

Bass and Cech observed the same activity of guanosine and 7-methylguanosine (7mG) (Fig. 1), a guanosine analog

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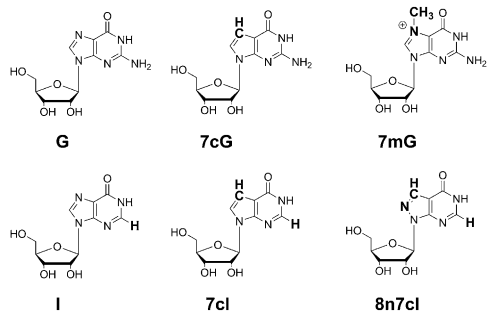

FIGURE 1. Guanosine analogs used in this work. Portions in bold represent changes relative to G.

that cannot accept coordination of a functional group to the N7, and concluded that the N7 atom was not involved in a functionally relevant interaction. However, subsequent work by Lin et al. (1994) showed that activity of the *Tetrahymena* intron decreases when 7-deazaguanosine (7cG) (Fig. 1) is used as the nucleophile. Thus, two different atomic mutagenesis probes to assess interactions at the same site gave distinct effects. Furthermore, one might have anticipated that introduction of the bulky methyl group and accompanying positive charge would be more likely to be disruptive, yet that analog was not perturbative, while the seemingly more conservative mutation of N7 to a C-H group was.

To explain the paradoxical results with 7mG and 7cG, Lin et al. (1994) proposed several models. In one model, the positive charge on the N7 of 7mG would fulfill the role of a metal ion normally interacting with N7 of guanosine. Alternatively, they suggested that N7 could take part in a water-mediated hydrogen-bonding interaction with one of the phosphoryl oxygens of the ribozyme's backbone; substitution of the N7 with a CH group would result in the loss of the contact with water, whereas the positive charge of 7mG could help to maintain the interaction with the negatively charged phosphoryl group. They also noted possible effects from changes in the charge distribution, stacking interactions, and hydrogen bond interactions of O6, N1, and N2.

Below, we describe tests of these models. Our results are consistent with a survey of prior observations and suggest that the 7-deaza substitution has effects that extend beyond removal of the nitrogen and its associated lone pair that must be considered when carrying out atomic mutagenesis experiments. These results also establish a powerful set of analogs to investigate the presence of atomic interactions at N7 of purines and stacking interactions with the purine ring.

RESULTS AND DISCUSSION

7-deazaguanosine, but not 7-methylguanosine, binds the ribozyme weaker than guanosine

The measurements of Lin et al. (1994) were carried out following the self-splicing reactions of the *Tetrahymena* group I intron. We made these measurements with a truncated version of the intron, the L-21 ScaI *Tetrahymena* ribozyme. This ribozyme catalyzes the site-specific attack of an exogenous guanosine (G) on a short oligonucleotide substrate (S) that mimics the 5′-splice site (Equation 1) in a reaction that corresponds to the first step of self-splicing. The ability to readily manipulate both substrates in the ribozyme reaction and the extensive kinetic and thermodynamic knowledge of individual reaction steps in the reaction of this ribozyme render it ideal for mechanistic investigations (Hougland et al. 2006).

$$\underset{S}{CCCUCUA} + G \rightarrow \underset{P}{CCCUCU} + GA \quad (1)$$

Table 1 shows the results obtained with G, 7mG, and 7cG (Fig. 1) in the ribozyme system and compares these results with those obtained previously for self-splicing. In the case of self-splicing, reactions were performed with an all-RNA system, whereas in our case the substrate contains a single deoxyribose substitution at position –1 to slow the reaction so that the chemical step is rate-limiting (Herschlag et al. 1993; McConnell et al. 1993). Thus, the maximal rates cannot be directly compared; however, apparent affinities of guanosine (and guanosine analogs) obtained under self-splicing correlate well with those obtained with the L-21 ScaI truncated version (e.g., see McConnell et al. 1993), suggesting that apparent binding affinities in self-splicing reflect guanosine binding and can be compared, at least in these cases, with binding constants obtained via pre-steady-state kinetics. Under our pre-steady-state reaction conditions, the concentration of G that provides half of the

TABLE 1. Binding and reactivity of exogenous G and G-analogs

	Single turnover			Self-splicing[a]	
	K_d (μM)	$k_c \times 10^2$ (min^{-1})	k_c/K_d[b] (M^{-1} min^{-1})	K_M (μM)	k_{cat} (min^{-1})
G[c]	52 ± 2	4.5 ± 0.3	660 ± 100 (1.0)	30 ± 12	0.25 ± 0.05
7mG[c]	27 ± 5	3.5 ± 0.1	800 ± 60 (1.2)	16 ± 3	0.40 ± 0.02
7cG[c]	1300 ± 90	5.8 ± 0.2	36 ± 8 (0.055)	450 ± 180	0.42 ± 0.05
I[d]	-	-	18 ± 1 (0.027)	-	-
7cI[d]	-	-	0.67 ± 0.05 (0.0010)	-	-
8n7cI[d]	-	-	17 ± 2 (0.026)	-	-

[a]Values from Lin et al. (1994). Errors were calculated from the reported kinetic parameters.
[b]Values in parenthesis represent the k_c/K_d value relative to that of G.
[c]Measured with S: −1d,rSA_5, 10 mM Mg^{2+}, 50 nM ribozyme (pH 6.9). Errors are from best fit of experimental data.
[d]Measured with S: −1d,rSA_5, 10 mM Mg^{2+}, 50 nM ribozyme (pH 8.3).

maximal rate, $K_{1/2}$, equals the true dissociation constant, K_d, as described previously (McConnell et al. 1993). Because the guanosine analogs shown in Figure 1 react with rate constants either similar to that of guanosine, or significantly slower than guanosine, the identity between $K_{1/2}$ and K_d likely holds also for these analogs. 7cG binds to the ribozyme–substrate complex ~25-fold weaker than G and 7mG, consistent with effects on apparent K_M values in self-splicing (Table 1; Lin et al. 1994). Once bound there is no significant difference in the observed reaction rate (less than twofold), and prior results strongly suggest that this rate reflects the chemical step (Herschlag and Koshla 1994; Knitt and Herschlag 1996).

Increasing concentrations of divalent metal ions have only a small effect on 7-methylguanosine binding

To explain the different binding affinities of G, 7mG, and 7cG, Lin et al. (1994) suggested that N7 of the exogenous G might be coordinated directly to a metal ion. In this case, replacement of the N7 with a CH would cause a decrease in the nucleophile binding affinity because of the inability of the CH group to coordinate to the metal ion, whereas the charge on the N7 of 7mG might mimic the positive charge of the native metal ion.

Structural models from the subsequent X-ray data do not support this hypothesis. The closest metal ion is 3.6–3.8 Å from the N7 of the exogenous G, too far for direct coordination, and is out of the plane of the guanine ring (Fig. 2; Adams et al. 2004; Stahley and Strobel 2005; Lipchock and Strobel 2008). We nevertheless carried out a functional test of this model. If the positively charged 7mG takes the place of a metal ion, competition for binding would be expected,

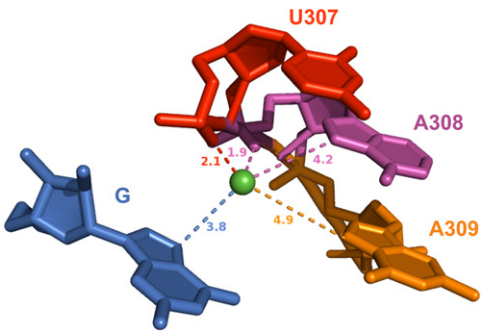

FIGURE 2. A metal ion near the exogenous guanosine (G) in group I ribozymes. Residues are numbered according to the *Tetrahymena* group I intron. Distances are in Å. This metal ion in green is present in models derived from crystals of the *Azoarcus* and Twort group I ribozymes (Adams et al. 2004; Golden et al. 2005; Stahley and Strobel 2005, 2007) but not in those derived from crystal of the *Tetrahymena* group I ribozyme (Guo et al. 2005), probably because of the particular truncation of the crystallized construct. The contacts between this Mg^{2+} ion and the *pro*-R_P phosphoryl oxygens of residues 307 and 308 are supported by functional studies on the *Tetrahymena* group I ribozyme (Forconi et al. 2007).

although the ion atmosphere surrounding the ribozyme could complicate such effects (Draper 2004; Das et al. 2005). Because the pK_a of the N1 atom of 7mG is 6.8 (Nishimura et al. 1980), we performed competition experiments at pH 5.9 and pH 6.9 to account for possible difference arising from the different protonation states of G and 7mG.

Regardless of the pH, the affinity of 7mG for the ribozyme decreases approximately twofold across a range of Mg^{2+} concentrations from 4 to 100 mM (Supplemental Fig. 1A), and the affinity of G for the ribozyme increases approximately twofold over the same range (Supplemental Fig. 1B). Thus, the affinity of 7mG for the ribozyme decreases about fourfold relative to that of G over a 25-fold range of Mg^{2+} concentration (Supplemental Fig. 1C). In contrast, direct competition with an active-site metal ion decreases the affinity of protonated 2′-aminoguanosine for the *Tetrahymena* group I ribozyme more than 25-fold over the same range of Mg^{2+} concentration (Shan et al. 1999). These results suggest a modest and likely indirect competition between the positive charge of 7mG and Mg^{2+} and do not support direct coordination of N7 of the exogenous G to a metal ion.

The 7-deaza modification does not perturb interactions on the Watson-Crick face

Another model suggested by Lin et al. (1994) was that introduction of the CH group at position 7 could disrupt other interactions between the nucleophile and the ribozyme. Such long-range effects have been observed in this system (Benz-Moy and Herschlag 2011; Forconi et al. 2011). For example, we recently showed that a 7-deaza substitution at the G264 position of the *Tetrahymena* ribozyme, a position that hydrogen bonds with the exocyclic amino group of the exogenous G (Fig. 3), causes the loss of multiple interactions between the G nucleophile and the ribozyme, possibly because of transmitted local rearrangements due to steric clashes with the 7-CH group (Forconi et al. 2011).

For the G nucleophile, the 7-deaza substitution could disrupt interactions on the Watson-Crick face of the guanine, which is involved in base-triple interactions with the ribozyme (Fig. 3; Michel et al. 1989; Adams et al. 2004; Golden et al. 2005; Guo et al. 2005; Stahley and Strobel 2005). To test whether interactions between the exocyclic amino group of the exogenous G and the ribozyme were compromised upon the 7-deaza substitution, we determined the effect of the 7-deaza substitution on binding and reactivity of inosine, a guanosine analog that lacks the exocyclic amino group (I) (Fig. 1). Because of weak binding of I (and 7-deazainosine, 7cI), we could only measure the second-order rate constant of the reactions (k_c/K_d), which reports on both binding of the nucleophile and reactivity once bound.

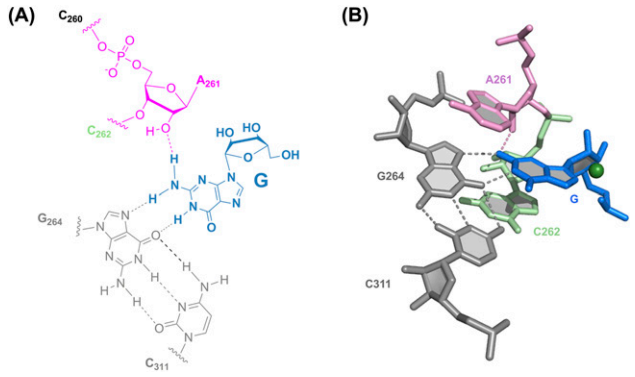

FIGURE 3. Hydrogen bonds and stacking interactions involving the exogenous guanosine. (*A*) Schematic of hydrogen bonding interactions between the base of the exogenous guanosine (shown in blue) and the *Tetrahymena* group I ribozyme from functional (Michel et al. 1989; Forconi et al. 2009) and structural (Adams et al. 2004; Golden et al. 2005; Guo et al. 2005; Stahley and Strobel 2005) data. (*B*) The same interactions are present in the *Azoarcus* group I ribozyme structural model 3BO3. The nucleobase of residue G128 (light green), corresponding to residue C262 in the *Tetrahymena* intron, seems to stack with the exogenous guanosine (blue). The Mg^{2+} ion shown in Figure 2 is also shown in this figure.

If interactions between the exocyclic amino group of the exogenous G and the ribozyme were lost, the 7-deaza substitution would be expected to give a larger effect on reaction of guanosine, in which the exocyclic amino group is present, compared with the effect on reaction of inosine, in which the exocyclic amino group is not present. Contrary to this prediction, there is a similar deleterious effect of 7-deazainosine (7cI) (Fig. 1) relative to I, as there is for 7cG relative to G (Table 1). Indeed, 7cI appears to be slightly more compromised, by approximately twofold, the opposite of the predicted effect. There is no indication of a significant and energetically costly rearrangement caused by the 7-deaza substitution.

Testing additional models: Introduction of a nitrogen atom at the 8-position

The results in the previous sections suggested the lack of a direct contact between N7 of the exogenous G and a divalent metal ion. However, it remained possible that N7 could take part in a water-mediated hydrogen bonding interaction with one of the phosphoryl oxygens of the ribozyme's backbone (Lin et al. 1994). According to this model, substitution of the N7 with a CH group would result in the loss of the contact, whereas the positive charge of 7mG would provide an electrostatic interaction with the negatively charged phosphoryl group without the need of the water molecule, although a significant rearrangement might be needed to bring a phosphoryl group in close proximity to the positive charge. Alternatively, a water molecule bound to N7 might directly or indirectly coordinate a metal ion, as suggested from examination of structural models of the ribosome (Sigel and Sigel 2010). Indeed, a metal ion is present near the N7 of the exogenous G (Fig. 2), although no associated water molecule is reported in the structural models. Replacement of the N7 with the CH group of 7cG would then likely perturb a putative water molecule interaction, whereas the positive charge of 7mG might allow structural and/or solvent rearrangements that coincidentally have no significant effect on binding, relative to G.

To contrast these models with possible more general electronic effects, we used an analog with a CH group at the 7-position, but with a nitrogen on the 8-position (8n7cI) (Fig. 1). In this analog, the bulky methyl group of 7mG is absent and the ability to directly coordinate a water molecule at the N7 position is abolished; a water molecule coordinated to the N8 would be >4 Å away from the metal ion shown in Figure 2, too distant to establish a direct contact. Nevertheless, many of the electronic properties of the base are maintained, relative to the unmodified nucleoside (Supplemental Table 1). We used 8n7cI instead of 8-aza-7-deaza guanosine because of its commercial availability, as guanosine and inosine behave similarly with respect to the 7-deaza substitution (Table 1).

As summarized in Table 1, 8n7cI reacts with the ribozyme with the same second-order rate constant as I, and 25-fold faster than the 7cI. This result suggests that there is no functionally important direct contact between the N7 of the nucleophile and a water molecule. Less likely is a network of water molecules surrounding the nucleophile that can readily rearrange to accommodate a contact with the nitrogen atom at position 8.

Duplex stabilities of the analogs correlate with the observed functional effects

To assess the origin of differences in the reactivity of analogs investigated herein that vary at the N7 position, we considered whether stacking interaction energies could differ with the different purines (Lin et al. 1994) and turned to literature data for the stability of nucleic acid duplex formation with these base analogs. Indeed, DNA duplexes containing a single 7-deazaguanine are 1–2 kcal/mol less stable than the unmodified DNA duplexes (Ganguly et al. 2007). In contrast, substitutions that did not diminish binding had little or no effect on duplex stability. DNA duplexes with a single 7-methylguanine were as stable as the unmodified ones (Ezaz-Nikpay and Verdine 1992), and those containing two 8-aza-7-deaza guanine residues were also similar in stability to the unmodified duplexes (0.3 kcal/mol more stable) (Becher et al. 2001). Differences in stacking and solvation could explain this trend, as discussed below.

The structure of the exogenous guanosine binding site in group I introns reveals apparent stacking interactions that sandwich the bound guanosine in addition to the hydro-

gen-bonding and metal-ion interactions with specific heteroatoms of the base and sugar (Adams et al. 2004; Golden et al. 2005; Guo et al. 2005; Stahley and Strobel 2005). In particular, residue C262 is located just below the exogenous guanosine, suggesting that stacking interactions with this residue, or with the C262–G312 base pair, are possible (Fig. 3B). Differences in global parameters such as dipole moment, quadrupole moment, and polarizability, have often been invoked to explain differences in stacking and cation–π interactions (Burley and Petsko 1985; Dougherty 1996; Mecozzi et al. 1996; Lai et al. 2003). Nevertheless, ab initio calculations for the guanosine analogs do not reveal any simple correlation between these parameters and the observed relative reactivity of the analogs (Supplemental Table 1), and visual inspection of electrostatic potential maps generated for these analogs also do not reveal striking differential properties associated with the measured functional effects (Supplemental Figs. 2, 3). Local effects involving the nitrogen atoms and the aromatic rings could contribute to stacking and thus binding (McKay et al. 2001; Wheeler 2011).

Differential solvation changes could also contribute to the 7cG effect. The Hoogsten face of guanosine is located near a cavity in the group I active site that can accommodate several water molecules (Adams et al. 2004; Golden et al. 2005; Stahley and Strobel 2005; Forconi et al. 2007), and recent studies have suggested that deaza substitutions in nucleobases are associated with significant water and cation loss (Ganguly et al. 2007, 2009, 2010).

Limited direct N7 contacts in group I ribozymes

The data presented above are in agreement with literature reports that suggest that the 7-deaza substitution alters several properties, such as stacking propensity and solvation (Ganguly et al. 2007; Wang et al. 2008; Shanahan et al. 2011), and imply that the decrease in binding affinity of 7-deazaguanosine is due to effects other than the loss of a specific contact with the N7. To further assess the functional effects from 7-deaza substitutions, we considered prior functional results with group I ribozymes in light of the available structural models. We focused on the *Tetrahymena* and *Azoarcus* group I ribozymes because of the existence of extensive functional and structural data. In particular, we used published structural models derived from crystals (Adams et al. 2004; Guo et al. 2005; Stahley and Strobel 2005; Lipchock and Strobel 2008) and functional data derived from nucleotide analog interference mapping (NAIM) experiments (Ortoleva-Donnelly et al. 1998; Strauss-Soukup and Strobel 2000) to consider whether inhibition of ribozyme activity observed upon 7-deaza substitutions arose from the loss of specific interactions.

NAIM experiments on the *Azoarcus* ribozyme identified seven sites at which 7-deazaadenosine is detrimental to ribozyme activity (Strauss-Soukup and Strobel 2000). Inspection of the available structural models reveals that each of these seven nitrogen atoms has a putative interacting partner (Table 2). In five cases it was possible to mutate this partner, but in only one case did this mutation lead to a detrimental effect. In the *Tetrahymena* ribozyme, NAIM experiments identified 10 7-deaza substitutions that slow the reaction (Table 3; Ortoleva-Donnelly et al. 1998). Only two of these sites are involved in a putative interaction, as judged by structural inspection and, in the only case for which the putative partner was mutated, no functional effect was observed.

While there are well-established examples of direct, functional N7 interactions (Michel et al. 1989; Wang et al. 1999; Forconi et al. 2011; Shanahan et al. 2011), the results presented herein show that functional effects upon removal of N7 do not necessarily indicate direct coordination to the 7-position, and the results at several other positions within group I introns are also consistent with indirect effects (Supplemental Table 2). Factors such as stacking interactions and solvation could contribute to the reduced activity of the 7-deaza purine analogs.

Here, we have shown that 7-methyl, 7-deaza, and 8-aza-7-deaza analogs represent a powerful set of compounds for distinguishing direct versus indirect interactions with N7 atoms. In case of direct interactions with N7, 7-methyl and 8-aza-7-deaza analogs should be as deleterious as, or more deleterious than, 7-deaza analogs. In case of indirect interactions, the reactivity of these analogs should be enhanced relative to that of 7-deaza analogs, as shown herein. The comparison between 7-methyl analogs and 8-aza-7-deza analogs can also provide information about the presence of a positive charge near the N7. Although most of these analogs are not currently available as phosphoramidites,

TABLE 2. Sites of 7-deaza interference in the *Azoarcus* group I ribozyme

Residue	Partner from crystal structure[a]	Interference in partner?[b]
A26	2′-OH G24	YES
A27	N2 G24	NO
A127	**H_2O 146**	**n.a.**
A129	N2 G128	NO
A136	**N6 A47**	**NO**[c]
A150	2′-OH A149	NO
A161	N3 U148	n.a.

From Strauss-Soukup and Strobel (2000). Residues in bold represent sites of 7-deaza interference also observed at corresponding positions of the *Tetrahymena* group I ribozyme.
[a]We used the first *Azoarcus* crystal structure (PDB entry: 1U6B) because its PDB file contains refined water molecules and metal ions. Partners do not change using more recent structural models, such as 3BO3.
[b]From Strauss-Soukup and Strobel (2000).
[c]NAIM result is from the *Tetrahymena* group I ribozyme (from Ortoleva-Donnelly et al. 1998).

TABLE 3. Sites of 7-deaza interference in the *Tetrahymena* group I ribozyme

Residue	Partner from crystal structure[a]	Interference in partner?[b]	Partner from Azo structure[c]
A95	NO	-	NO
A97	N3 U300	-	NO
A114	NO	-	N6 A87
A207	*NO*	-	*NO*
A256	NO	-	NO
A261	**NO**	-	**H_2O 146**
A270	**N6 A130**	**NO**	**N6 A47**
A301	NO	-	NO
A306	*NO*	*n.a.*	*H_2O 164*
A308	*NO*	*n.a.*	*NO*

From Ortoleva-Donnelly et al. (1998). Residues in bold represent sites of 7-deaza interference also observed at corresponding positions of the *Azoarcus* group I ribozyme. Residues in italics represent sites for which interference information could not be obtained for the *Azoarcus* group I ribozyme.
[a]Using the 1X8W structural model.
[b]From Ortoleva-Donnelly et al. (1998).
[c]We used both the first and the most recent structural models (PDB entries 1U6B and 3BO3, respectively). Contacts are identical regardless of the model, except for the lack of water molecules in the 3BO3 model.

given the power of this set of analogs, there is strong impetus to make them widely available.

MATERIALS AND METHODS

Materials

Tetrahymena L-21 ScaI group I ribozyme was prepared from in vitro transcription as previously described (Zaug et al. 1988). Guanosine, 7-methylguanosine, and inosine were purchased from Sigma-Aldrich. 7-deazaguanosine and 8-aza-7-deazainosine were from Berry & Associates, Inc. 7-Deazainosine was provided by the NIH/NCI open repository program. All nucleosides were used without further purification.

General kinetic methods

All cleavage reactions were single turnover, with 50 nM ribozyme in excess of the radiolabeled 5′-splice site analog (*S), which was always present in trace quantities. The 5′-splice site analog used was CCCUCdUAAAAA (-1d,rSA_5) to ensure that the chemical step was rate-limiting (Herschlag et al. 1993; McConnell et al. 1993). Reactions were carried out at 30°C in 50 mM buffer. The buffers used were Na-MES (pH 5.9), Na-MOPS (pH 6.9), and Na-HEPES (pH 8.3). Mixtures containing 10 mM $MgCl_2$, ribozyme (200–1000 nM), and 50 mM buffer (pH 6.9) were preincubated at 50°C for 30 min to renature the ribozyme. Aliquots were then withdrawn and added to tubes containing the appropriate amount of $MgCl_2$, buffer and nucleoside to reach the desired concentrations of each individual component. Reaction mixtures were allowed to equilibrate at 30°C for 15 min before the addition of *S to initiate the reactions. Reactions were followed by gel electrophoresis and analyzed as described previously (Herschlag and Cech 1990; Karbstein et al. 2002).

Measurements of kinetic parameters for reactions of guanosine and its analogs

To determine the kinetic parameters for reactions of the *Tetrahymena* L-21 ScaI ribozyme with exogenous guanosine (G), 7-methylguanosine (7mG) and 7-deazaguanosine (7cG), the rate of reaction of *S was determined with 0–2000 μM nucleophile (pH 6.9) (Na-MOPS) or 5.9 (Na-MES). The observed rate constant for cleavage of *S was plotted as a function of G, 7mG, or 7cG concentrations and fit to Equation 2.

$$k_{obs} = \frac{k_c\,[\text{nucleophile}]}{K_d + [\text{nucleophile}]} \quad (2)$$

Values of (k_c/K_d) were determined from linear fitting of the data at concentrations <20 μM.

Reactions of *S with inosine (I), 7-deazainosine (7cI), and 8-aza-7-deazainosine (8n7cI) were followed at 0–3500 μM nucleophile (pH 8.3) (Na-HEPES). No saturation was observed in these cases, and (k_c/K_d) values were determined from linear fits to the data.

Calculations

Theoretical calculations were performed with the Gaussian 03 software, revision E.01 (Frisch et al. 2004), using a standard density function theory and optimized at the B3LYP/6-311++G(d,p) level. Calculations were performed on N9-methylated nucleobases rather than on the full nucleosides.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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