Evidence That the Guanosine Substrate of the *Tetrahymena* Ribozyme Is Bound in the *Anti* Conformation and That N7 Contributes to Binding†

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ABSTRACT: The group I self-splicing introns are RNA enzymes that catalyze phosphodiester-exchange reactions. These ribozymes have a highly specific binding site for guanosine, a substrate for the first self-splicing reaction (Bass & Cech, 1984). The binding site for guanosine has been localized to a specific region of the ribozyme (Michel et al., 1989), but the conformation of the bound guanosine substrate remains unknown. Most analogs of guanosine with substituents at C8 have a preference for the *syn* conformation; however, some C8-substituted analogs have the potential to form a hydrogen bond between the C8 substituent and the 5′-hydroxyl that would stabilize the *anti* conformation; we have found that analogs with the potential to form such a hydrogen bond are more active substrates than those that cannot form such a hydrogen bond. These observations led us to test 8-5′-*O*-cycloguanosine, which is locked in the *anti* conformation, and 8-(α-hydroxyisopropyl)guanosine, which is locked in the *syn* conformation; the former is active as a substrate, while the latter is inactive. These results strongly suggest that guanosine is bound to the ribozyme in the *anti* conformation and provide an additional constraint on structural models of this RNA enzyme. We have also examined a series of N7-substituted guanosine analogs; this position had previously been assumed to be unimportant for substrate binding since 7-methylguanosine is an excellent substrate. However, we have found that 7-deazaguanosine and 7-methyl-7-deazaguanosine are less active substrates than guanosine. We discuss several models for the role of N7 in guanosine binding.

The *Tetrahymena* ribozyme uses guanosine (or GMP or GTP) as a substrate in the self-splicing reaction (Cech et al., 1981; Bass & Cech, 1984). The initial velocity of the splicing reaction as a function of guanosine concentration shows typical saturation kinetics, suggesting the existence of a specific binding site for guanosine. The functional groups involved in binding and/or catalysis, on the basis of the activities (k_{cat}/K_m) of guanosine analogs, are the keto (O6), amino (N2), and imino (N1) positions of the base, and the 2′- and 3′-hydroxyl groups of the ribose (Bass & Cech, 1984). Subsequently, a site on the ribozyme likely to be involved in a direct hydrogen-bonding interaction with N1 of the guanosine substrate has been localized to O6 of G264, a phylogenetically invariant nucleotide of the P7 helix common to all group I self-splicing introns (Michel et al., 1989).

In aqueous solution, guanosine 5′-monophosphate is distributed almost equally between the *syn* and *anti* conformations, in which the guanine base is either over or away from the ribose sugar (Son et al., 1972). Since use as a substrate by the *Tetrahymena* ribozyme requires binding interactions at both the base and the sugar and involves reaction with the 3′-hydroxyl of the sugar, the bound substrate is likely to be constrained to a particular conformation about the glycosidic bond. We have measured the substrate activities (k_{cat}/K_m) of a series of guanosine analogs which differ in their preferred (or allowed) conformations about the glycosidic bond as a probe of the conformation of the bound substrate. The structures of these analogs (Maeda et al., 1974; Holmes & Robins, 1964; Holmes & Robins, 1965; Stienmaus et al., 1971; Kohda et al., 1986) are shown in Figure 1A.

The N7 position of guanosine has not previously been considered to be an important site of interaction with the ribozyme because 7-methylguanosine is an active substrate; it was assumed that the methyl group on the N7 position would sterically interfere with binding if N7 was normally close enough to interact with the ribozyme (Bass & Cech, 1984). However, 7-methylguanosine is positively charged, and the resulting potentially favorable electrostatic interaction with the negatively charged sugar-phosphate backbone of the ribozyme might compensate for the loss of the normal interaction. We therefore tested a series of guanosine analogs with changes at N7 (7-methylguanosine, 7-deazaguanosine, and 7-methyl-7-deazaguanosine) to study the role of N7 in the guanosine–ribozyme interaction. The structures of these analogs (Ramasamy et al., 1988; Jones & Robins, 1963; Girgis et al., 1990) are shown in Figure 1B.

MATERIALS AND METHODS

Guanosine Analogs. Guanosine, 7-methylguanosine, and 8-bromoguanosine were obtained from Sigma; 8-aminoguanosine, 8-azaguanosine, 7-deazaguanosine, 7-methyl-7-deazaguanosine, and 9-deazaguanosine from R. K. Robins; 8-methylguanosine and 8-(α-hydroxyisopropyl)guanosine from D. Shugar; and 8-5′-*O*-cycloguanosine, 8-aminoguanosine, and 8-hydroxyguanosine from K. Kohda. Analogs were dissolved in water, loaded onto a C_{18} reverse-phase HPLC column, and eluted in 13 mM ammonium acetate, pH 5.2. Guanosine, 8-bromoguanosine, 8-methylguanosine, 8-aminoguanosine, 7-methylguanosine, 7-deazaguanosine, and 7-methyl-7-deazaguanosine were eluted in the same buffer with a 0–15% acetonitrile gradient over 30 min at a flow rate of 1 mL/min. One major peak was observed in all cases (except for our sample of 9-deazaguanosine, where 2 peaks were observed; only the material in one of these peaks had the correct UV spectrum). The material in each major peak was collected,

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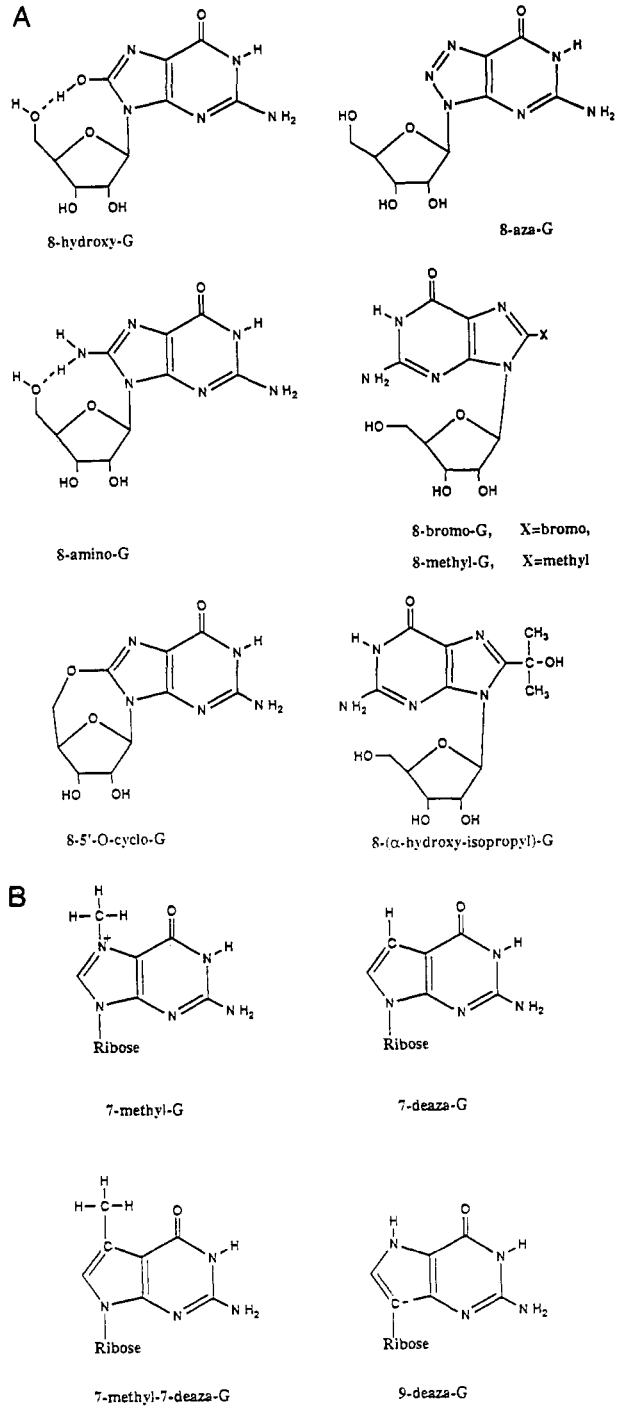

FIGURE 1: Guanosine analogs. (A) Analogs with varying conformational preferences. 8-Aminoguanosine, 8-hydroxyguanosine, and 8-azaguanosine are shown in the *anti* conformation; 8-methylguanosine and 8-bromoguanosine are shown in the *syn* conformation. 8-(α-Hydroxyisopropyl)guanosine is locked in the *syn* conformation. 8-5′-*O*-Cycloguanosine is locked in the *anti* conformation, with 3′-endo ribose puckering. (B) Analogs with N7 and N9 modifications.

dried, and redissolved in water. The identity of all analogs was confirmed by 300-MHz ^{1}H-NMR on a Gemini Varian spectrometer and/or FAB-MS. Mass spectra were obtained at the Harvard University Chemistry Department Mass Spectrometry Facility. Concentrations were determined from UV extinction coefficients taken from the literature.

RNA Transcription. *Tetrahymena* ribozyme labeled with [α-^{32}P]GTP was prepared by *in vitro* T7 run-off transcription of *Bam*HI-digested plasmid pSZ241 (Szostak, 1986). Transcription products were separated on a 6% polyacrylamide–8 M urea gel, visualized by UV shadowing, eluted into water overnight at room temperature, ethanol precipitated, and resuspended in water. This RNA consists of a 57-nucleotide 5′-exon, the entire 413-nucleotide intron, and a 44-nucleotide 3′-exon.

Measurement of Reaction Rates. We measured the rate of the self-splicing reaction of 5′-exon–intron–3′-exon in the presence of guanosine (or a guanosine analog) to yield free intron with a guanosine at its 5′-end plus ligated exons. These products result from two consecutive reactions: first, guanosine displaces the 5′-exon and becomes joined to the 5′-end of the intron, and second, the 5′-exon displaces the intron and becomes joined to the 3′-exon. However, under the reaction conditions used in this study, the second (exon-ligation) step is rapid, and the intermediates (free 5′-exon and intron–3′-exon) do not accumulate to any significant extent.

Gel-purified labeled *Tetrahymena* RNA was heated, in water, at 90 °C for 3 min in a heating block, after which 10X splicing buffer was added to a final concentration of 7.5 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.5, and 200 mM ammonium acetate (0.2 mM aurintricarboxylic acid was added as a ribonuclease inhibitor in some early experiments but had no effect on ribozyme activity). The solution was allowed to equilibrate at 25 °C for 15–30 min. The self-splicing reaction was initiated by mixing equal volumes of ribozyme and substrate solutions (guanosine or analog at various concentrations, also in 1X buffer); 5-μL aliquots were removed at intervals and mixed with 5 μL of 90% formamide, 25 mM EDTA to stop the reaction.

Reaction products were separated by electrophoresis on a 6% polyacrylamide denaturing gel. The radioactivity in each band was counted with a Betagen 2-D imaging detector. In each experiment the fraction of active ribozyme was determined from a 2–3-h splicing reaction with guanosine; the fraction of properly folded ribozyme, determined in this manner, varied from 0.5 to 0.8 between experiments. The velocity of the splicing reaction at each substrate concentration was obtained from the slope of the initial linear phase of a plot of product (ligated-exons, normalized to the fraction of active ribozyme) *vs* time. The splicing reaction was repeated at a series of substrate (guanosine or analog) concentrations to obtain a plot of velocity *vs* substrate concentration. This plot was directly fitted to the equation $V/E_T = (k_{cat}{}^*S)/(K_m + S)$ to obtain k_{cat}/K_m, K_m, and k_{cat}. Each set of kinetic data was obtained from a single experiment generating 28–30 data points, including both zero time and complete reaction (2–3 h) controls, and 6–9 time courses of splicing at different substrate concentrations.

Thin-Layer Chromatography. The purity of our HPLC purified 8-5′-*O*-cycloguanosine was assessed by loading 0.2, 0.4, and 2.0 nmol of guanosine, 0.28 nmol of HPLC purified 8-hydroxyguanosine, and 22.8 nmol of 8-5′-*O*-cycloguanosine onto five lanes of a fluorescent cellulose plate which was then developed with 2-propanol–1% aqueous ammonium sulfate, 3:2, v/v (Kohda et al., 1986). Under UV illumination, the spots due to 0.2 nmol of guanosine and 0.28 nmol of 8-hydroxyguanosine were visible, and the HPLC-purified 8-5′-*O*-cycloguanosine showed no contamination from either guanosine or 8-hydroxyguanosine. Therefore contamination of the 8-5′-*O*-cycloguanosine with either guanosine or 8-hydroxyguanosine, if any, was less than 1%. The 8-5′-*O*-cycloguanosine was well separated from guanosine and 8-hydroxyguanosine (R_f values: guanosine 0.52, 8-hydroxyguanosine 0.52, and 8-5′-*O*-cycloguanosine 0.37).

Table 1: Kinetics of Self-Splicing of the *Tetrahymena* Ribozyme with Guanosine and C8 and N7 Substituted Analogs

substrate	k_{cat},[a] min^{-1}	K_m,[a] μM	k_{cat}/K_m,[a] min^{-1} M^{-1}
guanosine	0.19 ± 0.04	25 ± 13	7300 ± 2600
	0.31 ± 0.05	34 ± 10	9200 ± 1500
	C8 Series		
8-bromo-G	0.26 ± 0.02	160 ± 26	1600 ± 140
	0.31 ± 0.02	130 ± 31	2400 ± 46
8-methyl-G	0.18 ± 0.01	96 ± 16	1900 ± 210
	0.41 ± 0.05	210 ± 75	1900 ± 480
8-amino-G	1.01 ± 0.04	160 ± 19	6200 ± 510
	0.85 ± 0.01	110 ± 8	7700 ± 430
8-hydroxy-G	0.48 ± 0.04	49 ± 9	9800 ± 1200
	0.75 ± 0.04	61 ± 10	12000 ± 1500
8-aza-G	0.83 ± 0.05	20 ± 3	41000 ± 4000
	0.68 ± 0.04	28 ± 7	24000 ± 3600
8-5′-*O*-cyclo-G	0.031 ± 0.001	45 ± 5	470 ± 92
	0.025 ± 0.001	49 ± 4	520 ± 31
8-AHI-G[b]		>3000[c]	4.4 ± 0.1[c]
		>3000[c]	3.5 ± 0.1[c]
	N7 Series		
7-methyl-G	0.21 ± 0.01	14 ± 3	15000 ± 3000
	0.60 ± 0.04	17 ± 3	35000 ± 4000
7-deaza-G	0.33 ± 0.02	390 ± 110	910 ± 190
	0.50 ± 0.08	510 ± 260	980 ± 400
7-methyl-7-deaza-G	0.47 ± 0.04	420 ± 280	1100 ± 600
	0.39 ± 0.03	540 ± 150	720 ± 160
9-deaza-G	0.003 ± 0.0002	140 ± 46	21 ± 6
	0.001 ± 0.0001	78 ± 24	11 ± 3

[a] Errors are standard errors from each experiment. The duplicate sets of numbers represent two independent experiments. [b] 8-(α-Hydroxyisopropyl)guanosine. [c] The velocity *vs* concentration plot for 8-AHI-G is linear up to a concentration of 3 mM, indicating that the K_m is greater than 3 mM. The slope, i.e. k_{cat}/K_m, is about 4 min^{-1} M^{-1}. It is not clear whether this residual activity is due to use of 8-AHI-G as a substrate or to contamination with some active nucleoside.

RESULTS AND DISCUSSION

The Glycosyl Torsional Angle. The 8-substituted analogs 8-methylguanosine and 8-bromoguanosine are found preferentially in the *syn* conformation due to steric hindrance between the 8-substituent and the ribose (Saenger, 1984). Both 8-bromoguanosine and 8-bromoadenosine crystallize in the *syn* conformation (Tavale & Sobell, 1970) as does 8-methyladenosine 3′-phosphate (Yasuniwa et al., 1979). In solution, less than 15% of 8-bromoguanosine is in the *anti* conformation (Pless et al., 1978; Lassota, 1984), and it has been estimated that only 20–30% of 8-methylguanosine is *anti* at any one time (Lassota, 1984; Stolarski et al., 1984). In contrast, 8-amino- and 8-hydroxy-substituted nucleosides are likely to exist predominantly in the *anti* conformation, due to the formation of a hydrogen bond between the 8-amino (or 8-hydroxy) and 5′-hydroxy groups. The proportion of 8-aminoguanosine in the *anti* conformation has been estimated to be about 60% (Lassota, 1984) and 8-(*N*-methylamino)-5′AMP, a related 8-amino nucleoside, is about 65% *anti* (Evans & Kaplan, 1976). We have found that k_{cat}/K_m for both 8-aminoguanosine and 8-hydroxyguanosine is about 4 times higher than for either 8-methylguanosine or 8-bromoguanosine (Table 1), consistent with a 2–4-fold increase in the fraction of substrate in the *anti* conformation. This difference in activity might also be attributed to the difference in the hydrophobicity of the substituents, which might favor the binding of 8-methyl- or 8-bromoguanosine if the interior of the ribozyme is hydrophobic, or disfavor their binding if the interior is strongly hydrophilic. Alternatively, if the ribozyme has a preference for the *anti* conformation of its substrate, the differences in the activities of these substrates may reflect their conformational differences. This hypothesis is consistent with the activity of 8-azaguanosine, in which the 8-CH is replaced with a nitrogen atom, and which exists predominantly in the high-anti conformation (Saenger, 1984); this analog is one of the few that we have identified that is actually more active as a substrate than guanosine itself.

The bromo, methyl, amino, and hydroxyl groups are similar in size and are not bulky enough to completely prevent rotation about the glycosidic bond (Saenger, 1984; Abdallah et al., 1975). In contrast, 8-(α-hydroxyisopropyl)guanosine is essentially locked in the *syn* conformation by the bulky C8-substituent (Pless et al., 1978), and 8-5′-*O*-cycloguanosine is locked by the covalent 8–5′ linkage into the *anti* conformation. We found that 8-(α-hydroxyisopropyl)-G is virtually inactive, whereas 8-5′-*O*-cycloguanosine is active as a substrate (Table 1). The very low k_{cat}/K_m of 8-(α-hydroxyisopropyl)-G (<0.1% that of guanosine) is consistent with a requirement that the guanosine substrate be bound to the ribozyme in the *anti* conformation; however, the very bulky group at the C8 position could simply prevent binding by steric hindrance. The relatively high k_{cat}/K_m of 8-5′-*O*-cycloguanosine (6% that of guanosine itself) is more significant. Because this analog is locked in the *anti* conformation, this result strongly suggests that it is the *anti* conformation of guanosine itself that is used by the *Tetrahymena* ribozyme. This result also implies that it is not necessary for the guanosine substrate to shift from *anti* to *syn* or vice versa during catalysis—the active site would have to be very flexible indeed for the *Tetrahymena* ribozyme to bind both the *syn* and *anti* conformations of its substrate, since both the base and the ribose of guanosine must interact with the ribozyme.

The above conclusions rely on the observed activity of 8-5′-*O*-cycloguanosine being due to 8-5′-*O*-cycloguanosine itself and not due to contaminating nucleosides. The most likely contaminants are 8-hydroxyguanosine and guanosine. The former could arise as a side product during synthesis or from hydrolysis during storage or during the splicing reaction, while the latter is the starting material for the synthesis. We used HPLC-purified 8-5′-*O*-cycloguanosine, which eluted as a single peak and showed no evidence of contamination. Analysis of a 100-fold excess of 8-5′-*O*-cycloguanosine next to guanosine and 8-hydroxyguanosine standards by TLC showed that there was less than 1% contamination by either of these compounds (see Materials and Methods). The kinetic data can also be used to argue that the observed activity of 8-5′-*O*-cycloguanosine is not due to contamination by either 8-hydroxyguanosine or guanosine. The apparent K_m for 8-5′-*O*-cycloguanosine is about 45 μM (Figure 2), at which the observed initial rate is 0.013–0.015 min^{-1}. Since it would require 5–10% contamination with 8-hydroxyguanosine or guanosine to achieve this rate if the 8-5′-*O*-cycloguanosine was inactive, we attribute the observed activity to 8-5′-*O*-cycloguanosine.

Why is the activity of 8-5′-*O*-cycloguanosine lower than that of guanosine? One possibility is that the 3′-hydroxyl group, which attacks the phosphate at the exon–intron junction, is not positioned properly for optimal catalysis, either because the glycosyl torsion angle χ of 8-5′-*O*-cycloguanosine is not the same as that of guanosine during catalysis or because the sugar conformation is different. The ribose of 8-5′-*O*-cycloguanosine may be locked in the 3′-endo conformation (Ikehara, 1969), while the ribozyme may prefer a 2′-endo ribose or require a flexible ribose to shift between the 2′-endo or 3′-endo conformations. Alternatively, steric hindrance due to the 8–5′ linkage over the sugar may impede substrate binding.

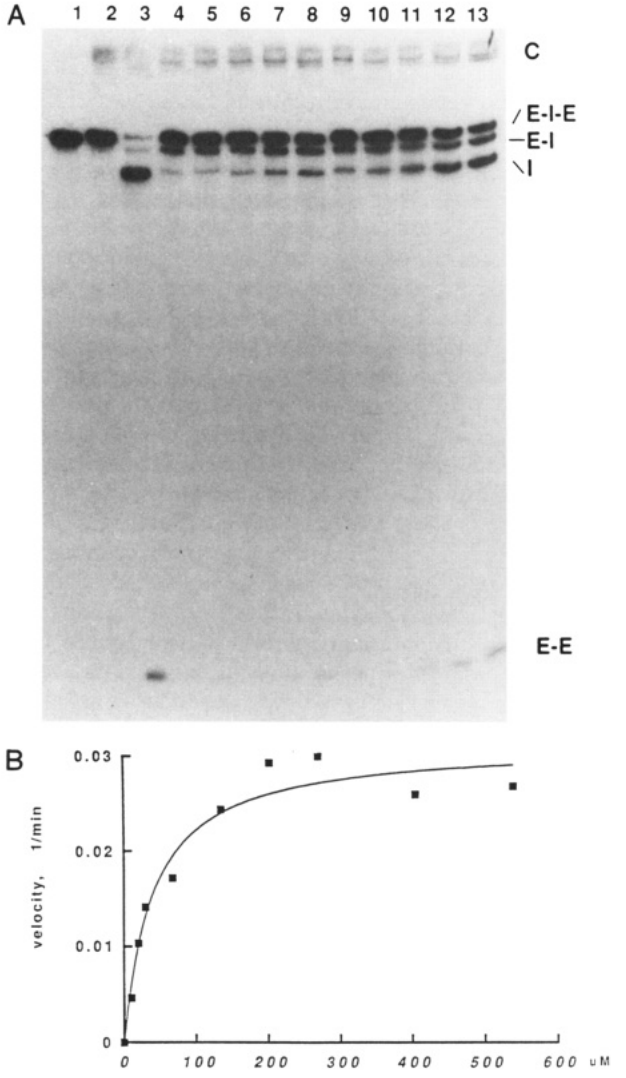

FIGURE 2: Self-splicing reactions with 8-5′-*O*-cycloguanosine. (A) Time courses of splicing with 8-5′-*O*-cycloguanosine. Reaction products were separated by electrophoresis on a 6% denaturing polyacrylamide gel. The bands from top to bottom are: circles (C), 5′-exon–intron–3′-exon (E–I–E, starting material), 5′-exon–intron (E–I, from hydrolysis of the 3′ splice-site), free intron (I), and ligated exons (E–E). The *Tetrahymena* ribozyme binds 8-5′-*O*-cycloguanosine and cleaves slowly at the 5′-exon–intron junction, followed rapidly by exon ligation. Therefore, the total reaction is the conversion of 5′-exon–intron–3′-exon to intron plus ligated exons, and the velocity is measured as the rate of accumulation of either ligated exons or free intron. Intron–3′-exon and 5′–exon, the products of cleavage at the 5′-exon–intron junction, are not detectable, because they are rapidly converted to free intron and ligated exons. Lane 1: unreacted starting material before heating. Lane 2: after heating to 90 °C for 3 min. Lane 3: 500 μM guanosine at 120 min showing the fraction of properly folded RNA, i.e., the active form. Lanes 4–8: 5 μM 8-5′-*O*-cycloguanosine at 5, 10, 20, 40, and 80 min. Lanes 9–13: 60 μM 8-5′-*O*-cycloguanosine at 5, 10, 20, 40, and 80 min. (B) Velocity *vs* concentration curve for 8-5′-*O*-cycloguanosine. The concentration *vs* velocity plot was directly fitted to the Michaelis–Menten equation to obtain k_{cat} = 0.031 min^{-1}, K_m = 45.5 μM, k_{cat}/K_m = 470 min^{-1} M^{-1}.

The conformation of the bound guanosine is an important constraint on the relative position and orientation of the P7 stem of the ribozyme (which interacts with the guanine base) with respect to the P1 stem, which interacts with the ribose 3′-hydroxyl. These constraints are important in defining the overall conformation of the ribozyme. Our results are consistent with the current three-dimensional model of the structure of group I introns (Michel & Westhof, 1990), which assumes that the guanosine substrate is bound in the *anti* conformation.

One may ask why all group I self-splicing introns use guanosine, and not adenosine or some other nucleoside, as a substrate (Been & Perrota, 1991). Since AMP exists largely in the *anti* conformation (Sarma et al., 1974), while GMP is almost equally distributed between the *syn* and *anti* conformations, it was previously suspected that the ribozyme might have taken advantage of that difference to impart added specificity to its substrate binding site. It now appears that this is not the reason for the use of guanosine. Indeed, a group I self-splicing intron can be mutated to form an adenosine-specific binding site (Been & Perrota, 1991), and recent experiments from this laboratory show that other RNA sequences can bind adenosine and adenosine nucleotides with high affinity and specificity (Sassanfar and Szostak, 1993), suggesting that the use of guanosine as a substrate for the group I introns may be the result of an accident of evolutionary history.

The N7 Position. We compared the activities of guanosine, 7-methylguanosine, and 7-deazaguanosine (Figure 1B) and were surprised to find that while 7-methylguanosine is at least as active as guanosine, 7-deazaguanosine is about 10 times less active than guanosine (Table 1). These nucleosides differ in a number of respects. The N7 of guanosine has been replaced by a CH group in 7-deazaguanosine, so that the 7-position now has a small hydrogen substituent, but remains uncharged. In contrast, 7-methylguanosine has a positively charged N7 with a large (methyl) substituent. In order to deconvolute the effects of these changes, we examined the activity of 7-methyl-7-deazaguanosine (Figure 1B), which has the large methyl substituent but no positive charge. The activity of 7-methyl-7-deazaguanosine is indistinguishable from that of 7-deazaguanosine. Thus, the positively charged N7 of 7-methylguanosine increases activity by 20–30-fold relative to 7-methyl-7-deazaguanosine, but moving from a small (hydrogen) substituent in 7-deazaguanosine to a large (methyl) substituent in 7-methyl-7-deazaguanosine has no effect.

One interpretation of these results is that the high substrate activity of 7-methylguanosine is due to its positive charge, which masks a 10-fold loss of activity which occurs when N7 is modified. The 10-fold decrease in activities of 7-deazaguanosine and 7-methyl-7-deazaguanosine relative to guanosine is consistent with the loss of a hydrogen bond to the ribozyme. For example, inosine, which lacks one or two hydrogen bonds between the amino group of guanosine and the ribozyme, is about 100 times less active than guanosine (Bass & Cech, 1984); 2-aminopurine riboside, which lacks one hydrogen bond from N1 position and one from O6, is also about 100 times less active than guanosine (Bass & Cech, 1984; Michel et al., 1989). However, it is unlikely that N7 is involved in a direct hydrogen bond with a donor on the ribozyme, since the required close contact would lead to a serious steric clash in the case of the 7-methylated analogs of guanosine. Since the activities of 7-deazaguanosine and 7-methyl-7-deazaguanosine are similar, a direct hydrogen bond involving N7 would require that the donor be on a part of the ribozyme flexible enough to accommodate the methyl group. We suggest that a more likely possibility is that the N7 interaction is indirect and is mediated by a water molecule or a metal ion. For example, N7 could take part in a water-mediated hydrogen-bonding interaction with one of the negatively charged phosphate oxygens of the backbone of the highly conserved J7/8 connecting region of the ribozyme, a region that lies close to the N7 position of the guanosine

substrate in the Michel–Westhof model (Michel & Westhof, 1990). In this model changing N7 to a CH (7-deazaguanosine) leads to loss of a hydrogen bond and a corresponding decrease in substrate activity; however, 7-methyl-7-deazaguanosine is no worse than 7-deazaguanosine because the methyl group can occupy the space previously occupied by the water, while the positive charge of 7-methylguanosine may interact favorably with the negative charges of nearby phosphate oxygens. A related possibility is that the N7 position is coordinated with a metal ion such as magnesium. In this case, the 7-deazaguanosine cannot coordinate the metal ion and is therefore less active; the methyl group of the methylated derivatives simply occupies the space normally occupied by the metal ion, and 7-methylguanosine is a good substrate because its positive charge plays the same role as the positive charge of the metal ion, without the entropic cost of localizing the metal ion. An alternative model is that changing N7 to CH causes electronic changes in the purine heterocycle that affect charge distribution, stacking interactions and/or changes in the strengths of the hydrogen-bonding interactions of O6, N1, and N2. Such electronic effects might also explain the high activity of 8-azaguanosine.

We sought an independent way of modifying N7 in order to confirm the effects noted above. N7 is protonated in certain guanosine analogs, such as 9-deazaguanosine (Figure 1B); surprisingly, this analog is 400-fold less active than guanosine in splicing. The low activity of 9-deazaguanosine, compared with 7-deazaguanosine and 7-methyl-7-deazaguanosine, may be due to the longer glycosyl bond (Prusiner et al., 1973). In general, the *C*-nucleosides, including 9-deazaguanosine, have a longer C–C glycosyl bond that allows the ribose to rotate more freely; for example, the glycosyl bond in formycin is 1.50 *vs* 1.46 Å in purine nucleosides. The imprecise location of the ribose, whose 2′- and 3′-hydroxy groups carry out the catalysis, may result in the slower reaction rate observed with 9-deazaguanosine.

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