Mutations at the guanosine-binding site of the *Tetrahymena* ribozyme also affect site-specific hydrolysis

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ABSTRACT

Self-splicing group I introns use guanosine as a nucleophile to cleave the 5' splice site. The guanosine-binding site has been localized to the G264-C311 base pair of the *Tetrahymena* intron on the basis of analysis of mutations that change the specificity of the nucleophile from G (guanosine) to 2AP (2-aminopurine ribonucleoside) (F. Michel et al. (1989) *Nature* 342, 391 – 395). We studied the effect of these mutations (G-U, A-C and A-U replacing G264-C311) in the L-21 *ScaI* version of the *Tetrahymena* ribozyme. In this enzymatic system (k_{cat}/K_m)^G monitors the cleavage step. This kinetic parameter decreased by at least 5×10^3 when the G264-C311 base pair was mutated to an A-U pair, while (k_{cat}/K_m)^{2AP} increased at least 40-fold. This amounted to an overall switch in specificity of at least 2×10^5 . The nucleophile specificity (G > 2AP for the G-C and G-U pairs, 2AP > G for the A-U and A-C pairs) was consistent with the proposed hydrogen bond between the nucleotide at position 264 and N₁ of the nucleophile. Unexpectedly, the A-U and A-C mutants showed a decrease of an order of magnitude in the rate of ribozyme-catalyzed hydrolysis of RNA, in which H₂O or OH⁻ replaces G as the nucleophile, whereas the G-U mutant showed a decrease of only 2-fold. The low hydrolysis rates were not restored by raising the Mg²⁺ concentration or lowering the temperature. In addition, the mutant ribozymes exhibited a pattern of cleavage by Fe(II)-EDTA indistinguishable from that of the wild type, and the [Mg²⁺]_{1/2} for folding of the A-U mutant ribozyme was the same as that of the wild type. Therefore the guanosine-binding site mutations do not appear to have a major effect on RNA folding or stability. Because changing G264 affects the hydrolysis reaction without perturbing the global folding of the RNA, we conclude that the catalytic role of this conserved nucleotide is not limited to guanosine binding.

INTRODUCTION

The nuclear pre-rRNA from *Tetrahymena thermophila* contains a 413 nt self-splicing intron (1,2). Splicing occurs by two transesterification reactions (2). In the first step, G (guanosine or one of its 5'-phosphorylated forms) acts as the nucleophile, attacking the 5' splice site and leaving the 5' exon with a 3'-hydroxyl terminus. In the second step, this 5' exon attacks at the 3' splice site, ligating the rRNA exons and releasing the intron.

Early work with guanosine analogues led to the proposal that the intron contains a saturable binding site for G (3–5). Michel et al. (6) subsequently localized this site to the G264-C311 base pair in the conserved paired region P7 (Fig. 1A). Evidence for the same location of the guanosine-binding site has been provided for two other group I introns, the terminal intron of the yeast mitochondrial cytochrome *b* pre-mRNA (7) and the bacteriophage T4 *sunY* intron (J. Doudna, pers. commun.). Michel et al. (6) showed that the specificity for the nucleophile could be switched from G to 2AP (2-aminopurine ribonucleoside) by changing this G-C base pair to A-U. Their data provided strong support for a hydrogen bond between the N₁-H of the nucleophilic G and the keto oxygen of G264 in the ribozyme and led them to propose that the nucleophilic G formed a base triple with the G264-C311 base pair (Fig. 1B). Yarus et al. (8,9) noted that the same data could be explained by models in which the incoming G is non-planar with G264-C311, and their studies with mutant ribozymes and alternative nucleophiles provided evidence for a contact between the G and A265 in the ribozyme (Fig. 1B). Furthermore, the base of C262 may stack on the base of the nucleophile, providing additional binding energy (10). The same guanosine-binding site also binds the 3'-terminal G of the intron for the second step of splicing (11), as predicted earlier (6,12).

Shortened forms of the *Tetrahymena* intron, such as the L-21 *ScaI* ribozyme (13), cleave exogenous RNA substrates with multiple turnover (14,15). Cleavage can occur by transesterification with G as the nucleophile, a reaction analogous to the first step of self-splicing. In the absence of G, cleavage occurs by hydrolysis with hydroxide ion or water as the

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nucleophile (16). The L-21 *ScaI* system allows the binding steps, the chemical step, and the product-release steps to be evaluated separately (16). Separation of the reaction into its individual steps is necessary for understanding the cleavage of mismatched substrates and of DNA (17,18), the effects of functional group substitutions (19,20) and the activity of mutant ribozymes (20,21). An additional advantage of the L-21 *ScaI* RNA is the absence of the 3'-terminal G of the intron, which could potentially occupy the guanosine-binding site and compete for the binding of exogenous nucleophile.

We now take advantage of the kinetic framework developed for the L-21 *ScaI* ribozyme to further explore the activity of the guanosine-binding site mutations of Michel et al. (6). In addition, we investigate the structural integrity of ribozymes with mutant guanosine-binding sites and the Mg^{2+} -dependence of their folding using the solvent-based Fe(II)-EDTA reaction (22–25).

MATERIALS AND METHODS

Mutagenesis. Site-directed mutants were made from pTZL-21 (26) according to the procedure of Kunkel et al. (27). Clones were screened by making mini-prep plasmid DNA (28) and dideoxy-sequencing the mutated regions (29). Plasmid DNA was prepared on a large scale using the boiling lysis procedure (30) and purified by ethidium bromide/CsCl density gradient centrifugation. Mutant plasmids were sequenced over the entire region that encodes L-21 *ScaI*. Mutants G264A, C311U, and the double mutant G264A:C311U are usually referred to as the A-C, G-U and A-U mutants, respectively, to designate the base combination present at positions 264–311.

Transcription of L-21 *ScaI* ribozyme. Plasmids were linearized with *ScaI* restriction endonuclease and transcribed as described by Zaug et al. (13). Purification of RNA included electrophoresis on a 4% polyacrylamide/8 M urea gel. Ribozyme concentrations were determined using $\epsilon_{260\text{ nm}} = 3.2 \times 10^6 \text{ M}^{-1} \text{ cm}^{-1}$ (13).

Synthesis and labeling of oligonucleotide substrate. The RNA substrate GGCCUCUAAAAA was transcribed from a synthetic DNA template by T7 RNA polymerase (31), 5'-end labeled and purified according to either of two protocols (13,19).

Kinetics. All rate constants were obtained from single turnover kinetics with $[E] \gg [S]$ as described previously (16). Briefly, reactions were carried out at 50°C in 50 mM MES sodium salt, pH 7.0 [determined at 25°C; pH 6.7 at 50°C, calculated from Good et al. (32)], and 10 mM $MgCl_2$ unless specified otherwise. Ribozyme (E) in $MgCl_2$ and MES buffer was preincubated 10–15 min at 50°C. For reactions with G or 2AP (Chemical Dynamics Corp.), the nucleophile was present during the preincubation period. For reactions not carried out at 50°C, the ribozyme mixture was preincubated at 50°C for 10–15 min, transferred to the temperature of the reaction and incubated for 5–7 min before addition of the oligonucleotide substrate. All reactions were initiated by addition of $\sim 1 \text{ nM}$ ^{32}P end-labeled oligonucleotide substrate (S). Typically, six aliquots were removed at specified times and quenched with ~ 2 volumes of a solution that contained 7 M urea or 90% formamide and 20 mM EDTA. Reaction products were separated by electrophoresis on 20% polyacrylamide/7 M urea gels, and the relative amount of substrate and product was quantitated using an Ambis

radioanalytic scanner. The disappearance of S followed first order kinetics, after correction for $\sim 5\%$ unreacted S. Reactions were typically followed for ~ 3 half-lives, with exception of the slow hydrolysis reactions, the slowest of which were determined by initial rate measurements. The k_{cat}/K_m values reported for the less reactive ribozyme-substrate combinations (G-C and G-U ribozymes with 2AP; A-U and A-C ribozymes with G) are expected to be due to reaction with the nucleoside indicated (9), although the possibility of reaction with a contaminating nucleoside has not been eliminated. Therefore, for these combinations the measured values of $(k_{\text{cat}}/K_m)^G$ or $(k_{\text{cat}}/K_m)^{2AP}$ must be considered to be maximum values. Because these maximum values appear in the denominator when calculating specificity ratios, the latter must be considered to be minimum values (note 'at least' in the Abstract).

Probing ribozyme tertiary structure by Fe(II)-EDTA cleavage. 5'-end labeled RNA was pre-incubated at 50°C for 10 min in 20 mM Tris buffer, pH 7.5, either in 10 mM $MgCl_2$ or without $MgCl_2$. It was then equilibrated at 42°C and treated with 1.0 mM $(NH_4)_2Fe(SO_4)_2$, 1.1 mM Na_2EDTA , 5 mM dithiothreitol for 80 min as described previously (25). The Mg^{2+} concentration dependence of tertiary structure formation was determined for the wild type and A-U mutant RNAs using 0–0.7 mM $MgCl_2$ (in 0.1 mM increments) at 30°C as described previously (25).

RESULTS

Nucleophile specificity switch

Site-specific mutagenesis was used to change the G264–C311 base pair to G-U, A-C and A-U base pairs in the L-21 *ScaI* form of the *Tetrahymena* ribozyme. The specificity of the wild type and mutant ribozymes for the identity of the nucleophile was determined by measuring k_{cat}/K_m at subsaturating concentrations of G (or the G analogue 2AP), using saturating concentrations of ribozyme (E) such that the reaction being monitored is that of the E·IS complex with the nucleophile. For the wild-type ribozyme there is evidence that $(k_{\text{cat}}/K_m)^G$ is at least partially limited by the actual chemical cleavage (16,19; D.H., F.Eckstein & T.R.C., manuscript in preparation). The wild-type ribozyme discriminated well between G and 2AP; the observed ratio of $(k_{\text{cat}}/K_m)^G/(k_{\text{cat}}/K_m)^{2AP}$ was $\sim 1 \times 10^3$ (Fig. 2A). The substitution of a G-U base pair at the guanosine-binding site led to a 10^3 fold reduction in activity with G, but maintained the specificity ($G > 2AP$). On the other hand, the substitution of A-C or A-U at the guanosine-binding site reversed the specificity: the k_{cat}/K_m value was then higher for 2AP than for G. The highest activity with 2AP occurred with the A-U mutant, but its $(k_{\text{cat}}/K_m)^{2AP}$ was still about 20 fold lower than the wild-type ribozyme's $(k_{\text{cat}}/K_m)^G$.

The effects of many mutations in group I introns are suppressed by elevated Mg^{2+} concentrations (33–36). The activities of the four ribozymes were therefore assessed as a function of Mg^{2+} concentration, each ribozyme being tested with its preferred nucleoside (Fig. 2B). The rate constants for the three mutant ribozymes increased only modestly with increasing $[Mg^{2+}]$, ~ 2 fold between 10 mM and 100 mM Mg^{2+} in the case of the G-U and A-C mutants and even less for the A-U mutant. The large deleterious effects of these mutations persist even at high Mg^{2+} concentrations.

Figure 1. Structure of the *Tetrahymena* ribozyme and proposed interactions at the guanosine-binding site. (A) Secondary structure of the L-21 *ScaI* ribozyme (solid line; ref. 54,55), with an RNA substrate (dashed line) bound to the substrate-binding site at the 5' end of the ribozyme to form paired region P1 (14). The critical G264-C311 base pair is boxed. The detailed view shows nucleotides in paired region P7 that help form the guanosine-binding site. The bound guanosine nucleophile (italic G_{OH}) attacks the cleavage site (diamond) of the bound substrate (lower case letters). The same local interactions are thought to occur in the endonuclease reaction catalyzed by the L-21 *ScaI* ribozyme and in the first step of RNA self-splicing. In the self-splicing reaction, the diamond would represent the 5' splice site and the 5' exon would end in the sequence cucucu. (B) Model of the guanosine-binding site. Hydrogen bonds with the guanosine or 2-aminopurine ribonucleoside (2AP) nucleophiles for which there is strong evidence from mutations that alter nucleophile selectivity are indicated by thick dashed lines (6,9). Hydrogen bond between the nucleophile and the N_7 of the base at position 264 (6) is stereochemically reasonable but has not been directly supported, and is therefore indicated by a dotted line in each structure.

Guanosine-binding site mutations affect ribozyme-catalyzed hydrolysis

The wild-type ribozyme also catalyzes the specific hydrolysis of the RNA substrate ($G_2CCCUCUpA_5 \rightarrow G_2CCCUCU + pA_5$) at the same site that is attacked by G (16). As in the G reaction, there is a thio-effect on $k_c(-G)$ (the rate constant for hydrolysis of bound RNA substrate by the wild-type ribozyme), consistent with the actual chemical reaction being at least partially rate-limiting for this rate constant (19). The A-C and A-U mutant ribozymes had a one order-of-magnitude reduction in $k_c(-G)$ (Fig. 3A). The Mg^{2+} -dependence of the hydrolysis rate constant was also determined (Fig. 3B). As with the guanosine reaction, the rate of the hydrolysis reaction exhibited an increase of about 2 fold between 10 mM and 100 mM Mg^{2+} with the least reactive ribozymes (in this case, the A-C and A-U mutants). The reaction rates of the G-U mutant and the wild-type ribozyme were essentially independent of $[Mg^{2+}]$ over this range. Although there was some restoration of hydrolysis activity for the A-C and

Figure 2. Ribozyme-catalyzed cleavage of an RNA substrate by attack of G and 2AP nucleophiles. (A) $(k_{cat}/K_m)^{nucleophile}$ values for each ribozyme with G (shaded) or 2AP (open) as the nucleophile (10 mM $MgCl_2$, 50 mM MES, pH 7, 50°C). The downward arrow represents a case in which no rate enhancement with added nucleophile was observed, so the indicated value is an upper limit. (B) The dependence of $(k_{cat}/K_m)^{nucleophile}$ on $[MgCl_2]$ for the reaction of the preferred nucleophile: G for the G-C and G-U ribozymes (● and ○, respectively) and 2AP for the A-U and A-C ribozymes (■ and □, respectively). $(k_{cat}/K_m)^{nucleophile}$ represents the rate constant for the reaction $E \cdot S + G$ or $2AP \rightarrow$ products, in a single turnover reaction ($[E] \gg [S]$, standard reaction conditions as described in the Methods). Data were obtained with the following concentrations of G and 2AP, respectively: G-C ribozyme, 2 and 1000 μM ; G-U ribozyme, 1000 and 1000 μM ; A-C ribozyme, 1000 and 20 μM ; and A-U ribozyme, 1000 and 20 mM. Rates for the hydrolysis reaction were determined in parallel reactions and were subtracted so that $(k_{cat}/K_m)^{nucleophile} = \{k_{obsd} - k_c(-nuc)\}/[nucleophile]$. The rate of each reaction was shown to be linear with respect to nucleophile concentration with 10 mM $MgCl_2$, indicating that ' $(k_{cat}/K_m)^{nucleophile}$ conditions' were attained. (Because of the small effect of $MgCl_2$ on the rates, these conditions presumably hold at all of the $MgCl_2$ concentrations.) $[Ribozyme] = 400$ nM; evidence that ribozyme is saturating under these conditions as described in the legend to Fig. 3.

A-U mutants at high Mg^{2+} concentration (e.g., the A-U mutant was 10 fold slower than wild-type ribozyme at 5 mM $MgCl_2$, and 4 fold slower at 100 mM), the defect in these ribozymes was not fully overcome.

At fixed Mg^{2+} concentration (10 mM), the hydrolysis reaction showed a marked temperature dependence (Fig. 4A).

Figure 3. Ribozyme-catalyzed hydrolysis of an RNA substrate for the wild-type and mutant ribozymes. (A) Comparison of $k_c(-G)$ under standard conditions (10 mM MgCl₂, 50 mM MES, pH 7, 50°C). (B) Dependence of $k_c(-G)$ on the concentration of MgCl₂. Ribozymes were ●, G-C; ○, G-U; ■, A-U; □, A-C. The rate constant $k_c(-G)$ represents the reaction: E·S → products, in a single turnover reaction ([E] > [S]), standard reaction conditions as described in the Methods). At 10 mM MgCl₂, the same rate constant was obtained with 100 and 400 nM ribozyme, for each ribozyme, indicating that the ribozyme is saturating. Reactions at the other MgCl₂ concentrations were carried out with 400 nM ribozyme.

Yet the values of $k_c(-G)$ relative to the wild-type ribozyme were essentially unchanged as the temperature was lowered from the standard 50°C to 15°C (Fig. 4B). Thus, lower temperature does not suppress the defects of the mutant ribozymes. From the temperature dependence of $k_c(-G)$, the thermodynamic parameters of activation associated with the hydrolysis reaction were calculated (Fig. 4C and Table 1). For the G-C, G-U and A-U ribozymes, the calculated values of $\Delta S^\ddagger$ at 50°C are positive, even taking into account the uncertainties of the values. (Because the uncertainties were based on maximum rather than average errors, they represent conservative limits.) A positive value of $\Delta S^\ddagger$ was also calculated for the A-C ribozyme, although in this case the error analysis does not preclude $\Delta S^\ddagger$ being near zero or slightly negative. The entropy of activation is of special interest because it provides an indication of the changes involved in attaining the transition state (see Discussion).

Figure 4. Temperature dependence of ribozyme-catalyzed hydrolysis. (A) Hydrolysis rate constants for reaction of 100 nM ribozyme and ~1 nM S in 10 mM MgCl₂, 50 mM MES, pH 7. Ribozymes were ●, G-C; ○, G-U; ■, A-U; □, A-C. (B) Hydrolysis rate constants for mutant ribozymes normalized to that of the wt ribozyme at the same temperature. (C) Arrhenius plot of the results presented in A. The slopes represent $-E_a/R$ and were calculated by linear regression analysis.

Structural analysis of guanosine-binding site mutants

RNA cleavage by free radicals produced by solvent-based Fe(II)-EDTA has proven to be a sensitive method for monitoring the three-dimensional structure of folded RNA molecules. In the presence of Mg²⁺ the wild-type ribozyme has a well-defined pattern of protected and exposed ribose sugars (23,37), and acquisition of this pattern occurs in a cooperative transition with a [Mg²⁺]_{1/2} of 0.8 mM at 42°C (25). Single base mutations and deletions in the ribozyme in several regions other than the guanosine-binding site have been found to alter the pattern of Fe(II)-EDTA protection or to increase the [Mg²⁺]_{1/2} for folding

Figure 5. Ribozyme tertiary structure probed by Fe(II)-EDTA cleavage. SM, starting material without incubation; all four ribozymes were similar. G, A, N, sequence ladders produced by partial digestion with RNase T₁ (which cleaves after G's), U₂ (which cleaves after A's), and alkali, respectively. + MgCl₂, 10 mM MgCl₂ included during incubation. Bands present in the - Fe(II)-EDTA lanes are due to Mg²⁺-catalyzed hydrolysis during incubation, so these positions were not evaluated in the + Fe(II)-EDTA + MgCl₂ experimental lanes.

(23; F. Murphy, B. Lagerbauer and T. R. C., unpublished results).

The guanosine-binding site mutants were analyzed at 42°C, 10 mM MgCl₂, a condition where they differ substantially in their ability to catalyze hydrolysis (Fig. 4A). No difference was observed in the Fe(II)-EDTA cleavage patterns (Fig. 5). The region of each molecule that could be assessed with high resolution (1–3 nucleotides) extended from the 5' strand of P4 through the 5' strand of P7. In regions past the 5' strand of P7 (beyond nucleotide ~270) the resolution decreased, but blocks of protected and accessible nucleotides were still distinguishable; again there was no apparent difference among the wild-type and mutant ribozymes. Both the wild type and A-U mutant ribozymes underwent the folding transition at the same MgCl₂ concentration (see Methods; data not shown). Thus, the Fe(II)-EDTA probe provided no evidence of perturbation of the folded state or of the stability of the ribozyme by the guanosine-binding site mutations.

DISCUSSION

We have applied some of the kinetic framework developed for the wild-type *Tetrahymena* ribozyme (16) to the guanosine-binding site mutants first described by Michel et al. (6). Changing the base pair involving nucleotides 264 and 311 from G-C to A-U decreased $(k_{\text{cat}}/K_m)^G$ by 6×10^3 and increased $(k_{\text{cat}}/K_m)^{2AP}$ by 40, giving a specificity switch of 2×10^5 . This is similar to the specificity switch of 1×10^5 reported by Michel et al. (6) for the first step in self-splicing also with subsaturating G and 2AP, but at a different temperature (30°C). (Both should be considered to be minimum values for the specificity switch; see Methods). The similarity in the values for the specificity switch is consistent with the same reaction step limiting the rate of both the endonuclease and self-splicing reactions with subsaturating nucleophile concentrations. Several types of data, including the measurement of the rate effect from a phosphorothioate substitution, are consistent with $(k_{\text{cat}}/K_m)^G$ being limited by the

Table 1. Thermodynamic parameters of activation for the hydrolysis reaction [$k_c(-G)$]

Ribozyme	$\Delta H^\ddagger$ (kcal/mol)	$\Delta G^\ddagger$ (kcal/mol)	$\Delta S^\ddagger$ (eu)
G-C	30 $\pm$ 4	22.0 $\pm$ 0.2	25 $\pm$ 13
G-U	32 $\pm$ 8	22.7 $\pm$ 0.2	29 $\pm$ 25
A-C	27 $\pm$ 5	23.8 $\pm$ 0.2	10 $\pm$ 16
A-U	31 $\pm$ 2	23.7 $\pm$ 0.2	23 $\pm$ 7

$\Delta H^\ddagger = E_a - RT$, with E_a from Fig. 4C and $T = 323$ K (50 °C).

$\Delta G^\ddagger = -RT \ln [h k_c(-G)/k_B]$, calculated using a standard state of the activity of $H_2O = 1$; R = gas constant, $T = 323$ K, h = Planck constant, k_B = Boltzmann constant.

$\Delta S^\ddagger = (\Delta H^\ddagger - \Delta G^\ddagger)/T$; 1 eu = 1 cal mol⁻¹ K⁻¹.

No correction was made for the change in pH of the MES buffer with temperature; given the pH dependence of $k_c(-G)$ (D.H., unpublished data), such a correction would make the $\Delta S^\ddagger$ slightly more positive. The uncertainty given for $\Delta H^\ddagger$ is an estimate of the maximum error determined as follows. For each ribozyme the line of steepest slope and the line of shallowest slope was determined using two of the four data points of Fig. 4C, and the values $E_a^{\max}$ and $E_a^{\min}$ were calculated. The error was then calculated as $(E_a^{\max} - E_a^{\min})/2$. The error for ΔG reflects a 30% standard deviation, which was the maximum standard deviation observed for the four ribozymes when multiple measurements (at least 9) of $k_c(-G)$ were performed independently. The error for $\Delta S^\ddagger$ is the sum of the errors for $\Delta H^\ddagger$ and $\Delta G^\ddagger$ divided by T .

chemical step for the endonuclease reaction with the wild-type ribozyme (16,19; D.H., F. Eckstein & T.R.C., in preparation). For self-splicing, no previous data indicate if the actual chemical step, some conformation change, or a mixture of these steps limits the rate under these conditions. We now suggest that the chemical step may also be rate-limiting for $(k_{cat}/K_m)^G$ in self-splicing.

In contrast, at saturating G, the much slower rate of self-splicing relative to the chemical step of the endonuclease reaction suggested that a conformational change limits the maximum velocity of self-splicing (16). Such a conformational change could also explain why a number of introns with mutations in and around the guanosine-binding site self-splice with a variety of nucleoside substrates at nearly equivalent maximal rates (8,9). Thus, it is suggested that there is a change in the rate-limiting step for splicing from the chemical step at low concentrations of G to a conformational change at higher concentrations of G.

New insights about the guanosine-binding site. The data of Fig. 2A support the earlier conclusion (6) that specificity is determined by the nucleotide in position 264. With a G-C or G-U pair at the guanosine-binding site, the ribozyme shows specificity for the guanosine nucleophile, but an A-C or an A-U pair switches the specificity to favor 2AP. The specificity switch holds over a wide range of Mg^{2+} concentration (Fig. 2B) and is consistent with the previously proposed hydrogen bond between O_6 of G264 and N_1 of the guanosine nucleophile (Fig. 1B). This may be one of a network of interactions between the nucleoside substrate and the ribozyme (Fig. 1B).

Two aspects of the data presented herein provide additional information about the guanosine-binding site. (1) In the simplest model for the guanosine-binding site, the only role of G264 would be to bind the guanine base of the guanosine nucleophile, thereby positioning guanosine for reaction. But the observation that changing G264 to A decreases the rate of hydrolysis in the absence of G ~ 10 -fold is contrary to this simple model (Fig. 3A). In addition, the smaller decrease in the hydrolysis rate for the G-U mutant than the A-U mutant, despite the greater

geometrical distortion expected from formation of the G-U wobble pair, suggests that G264 and not C311 is largely responsible for this effect. Thus, a structural and functional connection of G264 with other portions of the active site is suggested. It seems reasonable that G264, which is universally conserved among group I introns (38,39) and is packed within the catalytic core (23,37,39), could have more than one function. (2) Upon changing the conserved G-C pair to G-U, the rate of hydrolysis decreased only ~ 2 -fold, from which we argue that structural perturbation around the active site is minimal. In contrast, $(k_{cat}/K_m)^G$ decreased $\sim 10^3$ -fold. The substitution of a G-U wobble pair for the standard G-C pair would be expected to affect the position of G264 (40,41) and could also affect other local interactions. Our rate data then provide evidence that the G-C base pair is important for facilitating additional binding interactions with the guanosine or aligning the nucleophile for attack, or both.

The following results suggest local rather than global structural effects from the mutations in the guanosine-binding site. First, the A-U mutant binds the GGCCUCU oligonucleotide with a binding constant similar to that of the wild-type ribozyme (42), a value some 10^4 -fold tighter than expected for base-pairing alone and therefore indicative of tertiary interactions in the E $\cdot$ oligonucleotide complex (17,18,42–44). Second, at high concentration of G (or 2AP) and low concentration of S, where the wild-type ribozyme is limited by binding of S (ref. 16), the mutant ribozymes had rate constants within ~ 2 -fold that of the wild-type ribozyme (G-C and G-U with guanosine, and A-U and A-C with 2AP; data not shown). A significant population of improperly folded ribozymes incapable of binding S would result in a decreased rate constant (e.g., see $(k_{cat}/K_m)^S$ values in ref. 21). Furthermore, essentially all of S was cleaved with pseudo-first order kinetics. This rules out the presence of improperly folded ribozyme that binds S nonproductively such that it cannot be cleaved. Third, the same temperature dependence for the wild-type and mutant ribozymes in the hydrolysis reaction (Fig. 4B) is consistent with the mutants having the proper global structure. Finally, direct structural studies showed no difference between the wild-type and mutant ribozymes. Although it is quite possible that the Fe(II)-EDTA method might not have the sensitivity to detect some small structural change, particularly in the core region which is largely inaccessible to cleavage, mutations that cause the majority of RNA molecules to remain unfolded are easily recognized (23; F.Murphy and T.R.C., unpublished data).

The hydrolysis reaction. The observation that the guanosine-binding site mutations also affect the site-specific hydrolysis reaction is consistent with a single active site for cleavage by G and cleavage by H_2O (or OH^-). At the very least, there must be some interplay between the active site interactions responsible for these two reactions. In addition, the observation of a positive entropy of activation for the hydrolysis reaction by the wild-type and mutant ribozymes (Fig. 4C) indicates that the transition state is less ordered than the ground state for this reaction. This is unexpected, because the ribozyme-catalyzed hydrolysis reaction is thought to proceed by an $S_N2(P)$ mechanism (45,46), and nucleophilic displacement reactions of phosphate diesters and other compounds in solution typically give large negative values of $\Delta S^\ddagger$ (47–50). Because the value of $\Delta S^\ddagger$ represents the change in entropy for all of the steps in going from the ground state ($E \cdot S$) to the transition state ($[E \cdot S]^\ddagger$), the positive value of $\Delta S^\ddagger$ is still compatible with the chemical cleavage event being at least

partially rate-limiting in the hydrolysis reaction (19). The positive value of $\Delta S^\ddagger$ could reflect a conformational rearrangement and/or desolvation required to attain the transition state (51,52). One of many possibilities is that the oligonucleotide substrate becomes coordinated to an active site metal ion in the transition state (53), thereby desolvating the metal ion, which provides a positive entropic contribution, and activating the substrate.

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