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Mutations in a Nonconserved Sequence of the Tetrahymena Ribozyme Increase Activity and Specificity

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Summary

The RNA substrate-binding site of the Tetrahymena ribozyme is connected to the catalytic core by the joining region J1/2. Although J1/2 is not conserved among group I introns, small insertions or deletions in this sequence have dramatic effects, enhancing the turnover number and sequence specificity of ribozyme-catalyzed RNA cleavage. Measurements of rate constants for individual steps in the reaction have revealed the basis of these improvements. Ironically, the higher turnover and specificity both result from decreased affinity for RNA, rather than better cleavage. These results provide evidence that the nonconserved J1/2 sequence positions the RNA substrate to optimize tertiary interactions and ensure cleavage at the position corresponding to the 5′ splice site. The wild-type RNA is well adapted to its biological function, and its limitations in multiple turnover can be corrected by mutation.

Introduction

A ribozyme is an RNA molecule that folds to form an active site, promoting intramolecular catalysis (self-splicing, self-cleavage) or enzymatic activity (reaction on exogenous substrates with multiple turnover; reviewed by Cech, 1987, 1990; Altman, 1989; Pace and Smith, 1990). In the past few years, site-specific mutagenesis has been broadly applied to ribozymes to identify nucleotides involved in structure formation and catalysis and to define the minimum active unit (e.g., Price et al., 1985; Been et al., 1987; Jacquier and Michel, 1987; Waugh et al., 1989; Beaudry and Joyce, 1990; Couture et al., 1990; Michel et al., 1990; Ruffner et al., 1990). More than 300 mutations have been introduced in the Tetrahymena ribozyme, which cleaves RNA using G (guanosine or GTP) as a nucleophile (Zaug et al., 1986). Mutations in the RNA-binding site of this ribozyme change its sequence specificity for its RNA substrate (Waring et al., 1986; Been and Cech, 1986; Zaug et al., 1986; Murphy and Cech, 1989; see also Doudna and Szostak, 1989). Mutations in the G-binding site change specificity from guanosine to 2-aminopurine or adenosine (Michel et al., 1989; Been and Perrotta, 1991). Mutations outside these binding sites have generally appeared to be either neutral or deleterious to function under the conditions tested.

We have now found that small alterations in the L-21 ScaI form of the Tetrahymena ribozyme can result in dramatic enhancement of enzymatic activity. The region mutagenized was J1/2, which joins base-paired regions P1 and P2 (Figure 1). With saturating RNA substrate ("k_{cat} conditions"), the cleavage rate is increased up to 60-fold by the mutations. Furthermore, the same mutations greatly increase the ribozyme's specificity for a matched RNA substrate (one that makes a consecutive set of base pairs in helix P1; Figure 1a) relative to a mismatched substrate, by up to 70-fold. J1/2 is not conserved in length or sequence among group I introns, the larger family of which the Tetrahymena ribozyme is a member, and was omitted in the recent detailed structural model of Michel and Westhof (1990). This lack of conservation made the large effect of the J1/2 mutations particularly surprising.

Had we encountered these mutant ribozymes even 2 years ago, we would have had difficulty explaining their properties. We might have speculated that their higher values of k_{cat} reflected an improved catalytic center or the facilitation of some conformational change required for catalysis. Since that time, however, a kinetic description of the individual steps in the enzymatic cycle has been developed (Figure 2). Application of this kinetic framework to the J1/2 mutant ribozymes has provided simple explanations for their behavior. Ironically, their improved activity results from the disruption of the interaction between the ribozyme and its RNA substrate (S) and product (P) by the mutations. As shown in Figure 2b, the turnover number (k_{cat}) of the wild-type (wt) ribozyme is limited by slow product release (k_{off}^{P}); the mutations make the ribozyme a better enzyme by weakening binding, thereby accelerating product release. The wt ribozyme has limited specificity because it cleaves essentially every RNA molecule, matched or mismatched, that binds; the mutations increase specificity because weaker binding causes mismatched substrates to dissociate before reacting.

Furthermore, for two of the mutants, binding of RNA is weakened much more than binding of DNA of the same sequence. This observation provides a new type of evidence for the existence of two components of ribozyme–substrate association, base-pairing and tertiary interactions involving the substrate 2′-OH groups, in support of a model based on binding measurements (Sugimoto et al., 1989a; Pyle et al., 1990; Herschlag and Cech, 1990a, 1990b, 1990c; Pyle and Cech, 1991). We conclude that J1/2 contributes to the strength of substrate binding and to the proper alignment of bound substrate in the Tetrahymena ribozyme.

Results

Mutants Have Increased Turnover Number

The L-21 ScaI RNA, the form of the ribozyme used in previ-

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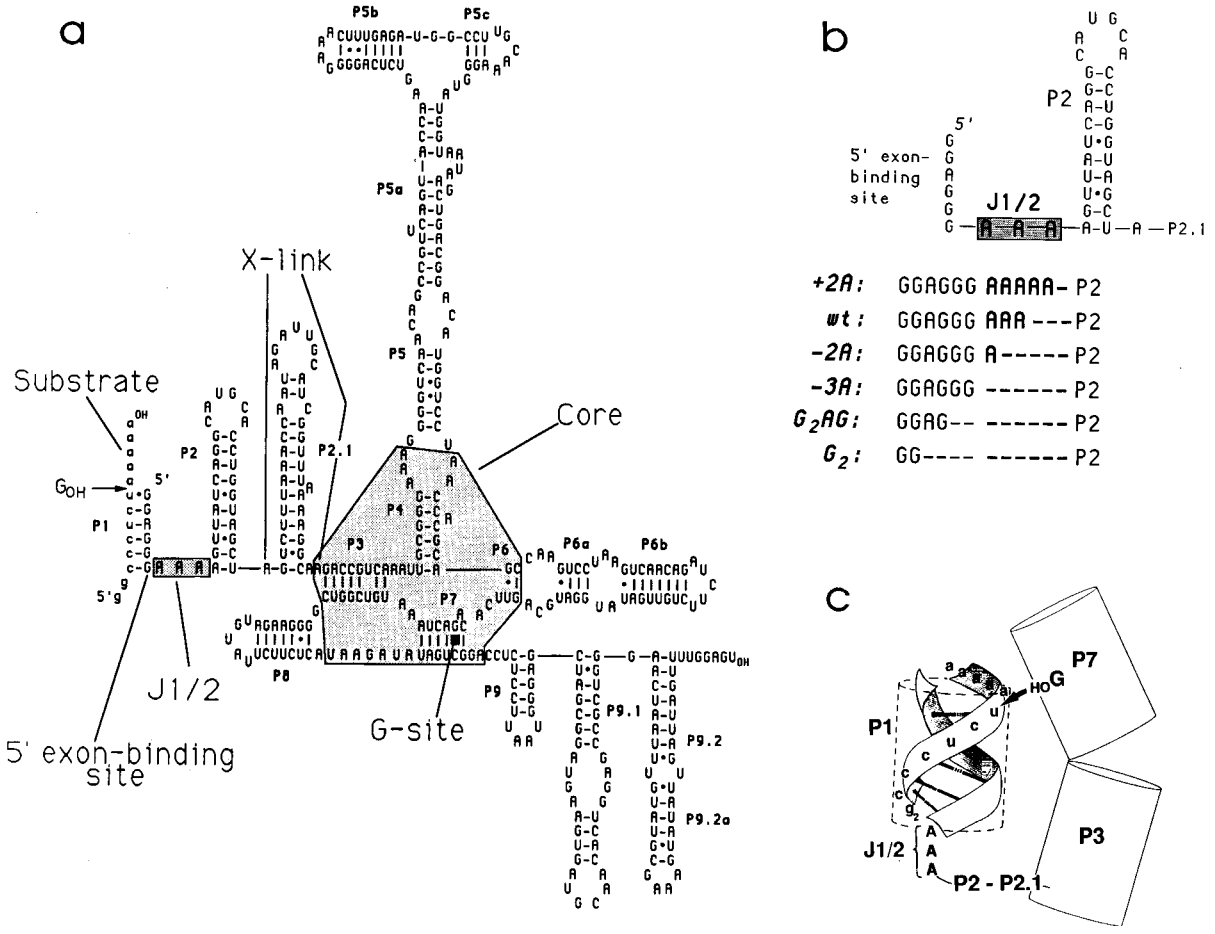


Figure 1. Structure of Tetrahymena Ribozyme, Showing Substrate-Binding Sites and Mutants Constructed

(a) The RNA substrate (lowercase letters) is shown bound to the 5′ exon–binding site of the L-21 ScaI ribozyme, forming paired region P1. Core, the catalytic core of the ribozyme, which includes the G-site (guanosine binding site) in P7 (Michel et al., 1989). X-link, an efficient UV cross-link that demonstrates juxtaposition of A57 and A95 in the folded structure (Downs and Cech, 1990).

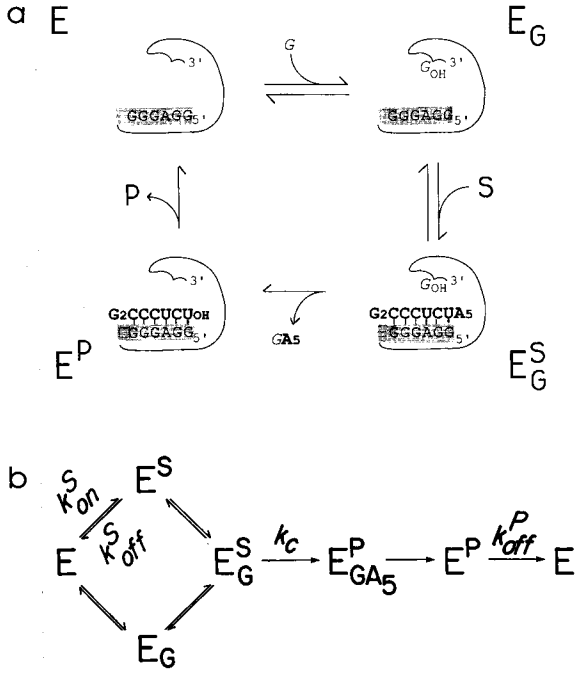
(b) The J1/2 region links the 5′ exon–binding site to the core. Deletion or addition of adenosine residues in this region by site-specific mutagenesis produced the +2A, −2A, and −3A ribozymes. Further deletion into the 5′ exon–binding site produced ribozymes named G_2AG and G_2. Dashes represent deleted nucleotides.

(c) A three-dimensional diagram useful for considering the effect of J1/2 mutations on positioning of P1 and on cleavage by guanosine. This model is consistent with the general architecture proposed by Michel and Westhof (1990); their model did not include J1/2.

ous mechanistic investigations, was mutagenized in the J1/2 region (Figure 1b). The +2A mutant has two A residues in addition to the three present in the natural (wt) molecule, while the −2A and −3A mutants are missing two or all three of these nucleotides, respectively. Mutant and wt ribozymes were assayed for RNA cleavage activity at 50°C in the presence of saturating GTP in 10 mM $MgCl_2$, 50 mM MES buffer (pH 6.7). With 0.5 μM matched RNA substrate, a concentration that is well above saturation for the wt ribozyme, the mutant ribozymes show dramatically enhanced activity (Figure 3, inset). Because the mutant ribozymes have higher K_m's for the RNA substrate (see below), and therefore are not all saturated at 0.5 μM, this experiment provides a lower limit for the turnover number, k_{cat}. The actual values of k_{cat} are listed in Table 1; the −2A mutant has the highest value, approximately 60 times that of the wt ribozyme.

Product dissociation (k^P_{off}, Figure 2b) is rate limiting for RNA cleavage by the wt ribozyme under conditions of saturating S (i.e., $k_{cat} = k^P_{off}$). Thus, any mutant with an increased k_{cat} must have an increased k^P_{off} (Herschlag and Cech, 1990a). In agreement with this expectation, all three mutant ribozymes show weakened interaction with P (Table 1, K^P_d).

The rate-limiting step for k_{cat} for the mutant ribozymes could have been k^P_{off}, as in the case of the wt enzyme, or there could have been a change in the rate-limiting step for the mutants. As shown in Figure 3, k_{cat} increases proportionately to K^P_d upon introduction of the +2A and −2A mutations; the slope of the line is equal to unity, within experimental error. Such proportionality is expected if product release remains rate limiting, because $K^P_d = k^P_{off}/k^P_{on}$; and k^P_{on} is likely to be constant, because k^S_{on} is nearly constant (see following section). The value of k_{cat} for the



Ribozyme	k_{on}^S (10^8 M^{-1} min^{-1})	k_{off}^S (min^{-1})	k_c (min^{-1})	k_{off}^P (min^{-1})
wt	1.0	0.2	700	0.1
+2A	0.8	1	300	1
-2A	1.5	20	400	6
-3A	0.05	10	400	3

Figure 2. Cleavage of RNA by the wt Ribozyme

(a) Ribozyme-catalyzed cleavage of a "matched" RNA substrate S (GGCCCUCUAAAAA), which forms a continuous set of base pairs with the 5′ exon–binding site (shaded) of the ribozyme. E, ribozyme; E_G, ribozyme with guanosine bound; E^S_G, ternary complex; E^P, ribozyme with bound product P (GGCCCUCU).

(b) Kinetic scheme from Herschlag and Cech (1990a). S and G undergo unordered and independent binding to the ribozyme. The resulting ternary complex reacts very rapidly with rate constant k_c. One of the products, GA_5, is released very quickly. Release of product P with rate constant k_{off}^P is rate limiting under k_{cat} conditions. The numbers in the table are derived from Table 1: $k_{on}^S = (k_{cat}/K_m)^S$, $k_{off}^S = k_{on}^S K_d^S = k_{on}^S K_d^P$ for the mutant ribozymes (wt value from Herschlag and Cech, 1990a), and $k_{off}^P = k_{cat}$; justification of these relationships is given in the text and in the Experimental Procedures.

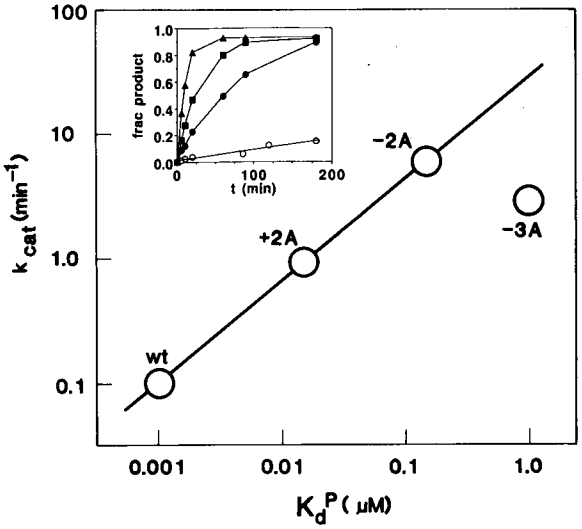


Figure 3. Mutants Have Greatly Increased Turnover Number (k_{cat})

Relationship between k_{cat} and K_d^P for the wt and mutant ribozymes (values from Table 1). Inset shows sample of primary data: reactions contained 0.5 μM RNA substrate (5′-^{32}P-GGCCCUCUAAAAA), 500 μM GTP, and 10 nM ribozyme (open circles, wt; closed circles, +2A; closed triangles, −2A; closed boxes, −3A).

−3A mutant falls below the line, consistent with the incursion of an additional step for this mutant (see separate section below).

For the +2A mutant, a burst of product formation preceded a slower steady-state accumulation of product (data not shown). This observation independently shows that the rate-limiting step for steady-state turnover occurs after cleavage (as described for the wt ribozyme by Herschlag and Cech, 1990a).

Reaction Rate with Subsaturating S Not Affected by +2A and −2A Mutations

The second-order rate constant $(k_{cat}/K_m)^S$ measures the rate of reaction of E_G and free S. (E_G indicates the ribozyme with guanosine bound but with its RNA substrate–binding site unoccupied; S is the RNA substrate.) For the +2A and −2A variants, this kinetic parameter was equal to that of the wt ribozyme within experimental error (Figures 4a and 4b). Because they have similar values of $(k_{cat}/K_m)^S$ and much higher values of k_{cat}, these mutant ribozymes cleave RNA as fast or faster than the wt ribozyme at all concentrations of E and S as long as G is saturating.

There was a sharp decrease in $(k_{cat}/K_m)^S$ for ribozymes with larger deletions (Figure 4b). The G_2AG and G_2 mutants are missing one-third or two-thirds of the substrate-binding sequence. Thus, the surprise for these mutants is not so much that $(k_{cat}/K_m)^S$ is decreased, but that so much activity remains. The G_2 mutant ribozyme reacts ~100-fold faster with GGCCCUCUA$_5$ than with GGCCCGCUA$_5$ (200 nM ribozyme, 400 μM GTP, and ~1 nM 5′ end–labeled oligonucleotide substrate; data not shown), suggesting that a base pair with the underlined U is maintained even though its normal pairing partner in the 5′ exon–binding site has been deleted. This result is consistent with the first base of P2, an A residue, being recruited for the 5′ exon–binding site. The similarity of $(k_{cat}/K_m)^S$ values for the G_2AG and G_2 mutants gives rise to the possibility that in these mutants the A residue that begins P2 and perhaps even the G following it are free rather than base-paired as depicted in Figure 1a. (It should be noted that base-pairing at the base of P2 is not proven for the wt ribozyme.) However, it remains possible that a ribozyme sequence not involving P2 acts as a surrogate 5′ exon–binding site.

For the wt ribozyme, the rate-limiting step under $(k_{cat}/K_m)^S$ conditions is substrate binding. This was demonstrated by pulse–chase experiments: essentially every RNA substrate molecule bound in E^S_G proceeded to react rather than to dissociate (Herschlag and Cech, 1990a;

Table 1. Kinetic Constants for Cleavage of $G_2CCCUCUA_5$ by Wild-Type and Mutant Ribozymes

Ribozyme	k_{cat} min^{-1}	K_d^P μM	K_d^{dP} [a] μM	$(k_{cat}/K_m)^S$ $10^8 M^{-1}min^{-1}$	$(k_{cat}/K_m)^G$ $10^5 M^{-1}min^{-1}$	K_d^G [b] mM	k_c [b,c] min^{-1}	$k_c(-G)$ [d] min^{-1}
wt[e]	0.1	0.001	20	1.0	7	1	700	0.6
+2A	1	0.015	150	0.8	0.8	(4)	(300)	0.2
−2A	6	0.15	30	1.5	1.0	(4)	(400)	0.1
−3A[f]	3	1.0	110	0.05	∼1	(4)	(∼400)	∼0.4

All reactions in 50 mM MES (pH 6.7) and 10 mM $MgCl_2$ at 50°C. P = GGCCCUCU, dP = d(CCCUCU). Conditions for determination of the individual kinetic parameters are described in the Experimental Procedures. Except where noted below, the precision of the rate and equilibrium constants is better than 20% with reactions performed side by side. Experiments performed on different days with different solutions varied as much as 2-fold. Wild-type and mutant ribozymes were always compared side by side.

[a] Note that the relative values are more accurate than the absolute values, as reactions were performed side by side with the same dP solutions.

[b] Parentheses denote that the values depend on the assumption that the wild-type and mutant ribozymes have roughly the same temperature dependence for K_d^G (see Experimental Procedures) and that there is relatively large uncertainty in these values.

[c] The rate constant for the chemical step $k_c = (k_{cat}/K_m)^G \times K_d^G$. Data supporting this relationship is presented in Herschlag and Cech (1990b) and Herschlag et al. (1991).

[d] Rate constant for the site-specific hydrolysis of ribozyme-bound S* in the absence of G (Herschlag and Cech, 1990a).

[e] From Herschlag and Cech (1990a, 1990c). All values were confirmed in side-by-side experiments with the mutant ribozymes in the present study.

[f] Values preceded by "∼" are more uncertain because the measured value was corrected using an estimated value of K_d^G (see Experimental Procedures).

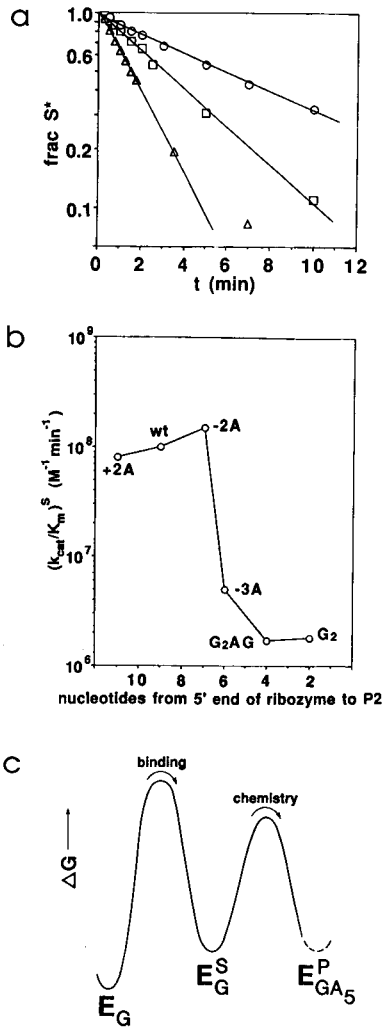


Figure 4. $(k_{cat}/K_m)^S$, the Second-Order Rate Constant for Reaction of E_G and S, for wt and Mutant Ribozymes

(a) Example of data used to determine $(k_{cat}/K_m)^S$. Single-turnover experiments with ∼1 nM S and 500 μM GTP were performed with the −3A ribozyme. [E] = 30 nM (open circles), 50 nM (open boxes), and 90 nM (open triangles).

(b) Values of $(k_{cat}/K_m)^S$ for wt and mutant ribozymes (Table 1). $(k_{cat}/K_m)^S = k_{obs}/[E]$, with k_{obs} determined from the slopes of lines from experiments similar to that shown in (a).

(c) Free energy diagram for reaction of free S with the E_G complex for the wt ribozyme, illustrating that binding of S is the rate-limiting step under subsaturating S or "$(k_{cat}/K_m)^S$ conditions" (Herschlag and Cech, 1990a).

E_G^S is the ternary complex of ribozyme, guanosine, and RNA substrate). Thus, for the reaction of E_G + S, the binding of S must be the highest energy barrier along the reaction coordinate so that $(k_{cat}/K_m)^S = k_{on}^S$ (Figure 4c). The +2A and −2A ribozymes have values of $(k_{cat}/K_m)^S$ similar to that of the wt, suggesting that k_{on}^S is also rate limiting for these mutant ribozymes. Pulse–chase experiments with the +2A and −2A ribozymes confirmed that k_{on}^S is indeed rate limiting; essentially all bound S reacted to give P (data not shown), as with the wt ribozyme. A conformational change associated with binding appears to slow $(k_{cat}/K_m)^S$ for the −3A ribozyme, as described in a separate section below.

Increased Specificity

The type of specificity that is of particular interest for ribozymes is the ability to discriminate against RNA molecules with nucleotide sequence similar but not identical to that of the cognate substrate. Such substrates (S_{MM}) form a mismatched duplex when they pair with the substrate-binding site of the ribozyme, in contrast to the continuous stretch of base pairs formed by the matched substrate (S_M). When matched and mismatched substrates compete for the same ribozyme, the specificity (relative rate of reaction of two substrates present at equal concentration) is determined by the ratio of values of $(k_{cat}/K_m)^S$ (Figure 5a). This equation holds independent of whether the substrate

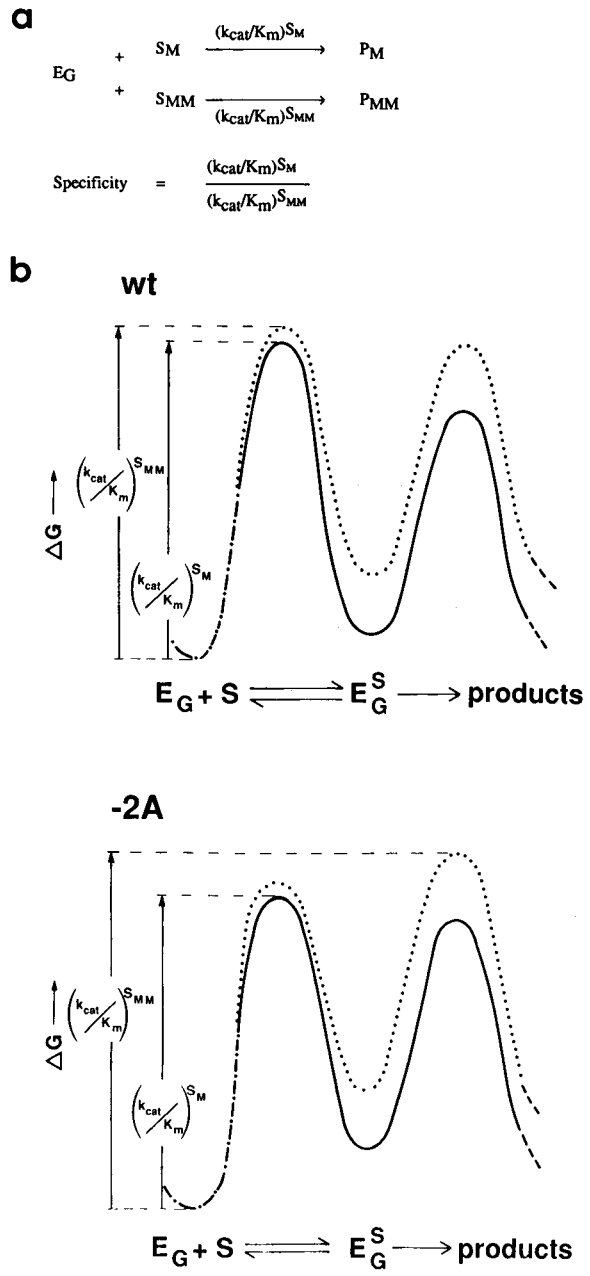


Figure 5. Basis for the Expectation of Increased Specificity with Mutant Ribozymes

(a) Definition of ribozyme specificity, the relative rate of cleavage of the matched RNA substrate S_M and a mismatched substrate S_{MM} present at equal concentration. If the concentrations were unequal, the specificity equation would include an additional factor $[S_M]/[S_{MM}]$. The particular substrates used in the experiments and calculations described here are S_M = GGCCCUCUA$_6$ and S_{MM} = GGCCCGCUA$_6$, where underlining indicates position of mismatch.

(b) Free energy reaction profiles for wt and −2A ribozymes under "$(k_{cat}/K_m)^S$ conditions," in which there is saturating guanosine and subsaturating (0.1 nM) S_M or S_{MM}. Calculations are described in Experimental Procedures.

wt: For the matched substrate (solid line), the activation energy barrier for binding is significantly higher than that for the chemical step, so essentially every substrate molecule that binds is cleaved. For the mismatched substrate (dotted line), the activation energy barriers for binding and for the chemical step are of similar height, so that these two steps are each partially rate limiting; nevertheless, about 70% of the mismatched substrate molecules that bind go on to react. Thus, although there is a large difference in K_d between matched and mismatched substrates, the specificity is low.

-2A: The −2A deletion in J1/2 destabilizes the E_G^S ground state. Additional destabilization caused by a mismatch in the binding interaction is expected to change the rate-limiting step from binding to chemistry. Because the activation energy barrier for the chemical step is higher than that for substrate dissociation, the substrate has a chance to attain binding equilibrium with the ribozyme prior to cleavage. This would allow the ribozyme to discriminate against S_{MM}.

concentrations are saturating or subsaturating (Fersht, 1985). The tight binding of RNA substrates to the wt ribozyme results in cleavage of essentially every RNA molecule that binds, whether the substrate is matched (S_M = GGCCCUCUA$_6$) or mismatched (S_{MM} = GGCCCGCUA$_6$), despite the fact that the mismatched substrate binds more weakly by a factor of 1200 ($K_d^{S_{MM}}/K_d^{S_M}$ = 2.5 μM/2 nM = 1200; Herschlag and Cech, 1990a, 1990b). Thus, the specificity of the wt ribozyme is only ~5 under standard reaction conditions (Figure 5b, wt). We expected that weaker binding to the mutant ribozymes would provide increased discrimination between the matched and mismatched substrates (Figure 5b, −2A).

The prediction of higher specificity of the mutant ribozymes was tested by determining their activity with the matched and a mismatched substrate under $(k_{cat}/K_m)^S$ conditions. Qualitatively, it is apparent from Figure 6a that the wt ribozyme cleaves S_M preferentially to S_{MM} but also gives substantial cleavage of the mismatched substrate even at early reaction times. In contrast, the −2A mutant is able to cleave the matched substrate completely under conditions in which no cleavage of the mismatched substrate is visible. Also apparent in Figure 6a is the decreased fidelity of cleavage of both S_M and S_{MM} by the mutant ribozyme. We use the term "fidelity" to describe the accuracy with which the cleavage site is chosen, while "specificity" describes the competition of different substrate molecules for cleavage by the ribozyme.

To quantitate the specificity difference, $(k_{cat}/K_m)^S$ was determined for the matched and mismatched substrates with wt and mutant ribozymes. As summarized in Figure 6b, specificity increased from ~10 with the wt ribozyme to ~700 with the −2A variant. The maximum difference in $(k_{cat}/K_m)^S$ expected for this pair of substrates is ~2000, calculated as follows. The K_d values of the two substrates differ by 1200, as described above. In addition, a mismatch slightly slows the chemical step, giving an additional factor of 700 min^{-1}/400 min^{-1} = 1.8 (Table 1 and Experimental Procedures). Thus, the maximum discrimination expected would be 1200 × 1.8 ≈ 2000. The specificity observed for the −2A ribozyme approaches this value within a factor of ~3. The specificity of the −3A mutant is similar to that of the −2A mutant, despite its higher K_d, so it falls off the correlation in Figure 6b. The explanation is that the higher K_d results from an inactive conformation of the free -3A ribozyme, which weakens the observed binding (see separate section below). Because individual rate constants for the active -3A ribozyme are similar to those for the −2A ribozyme, the specificity is similar.

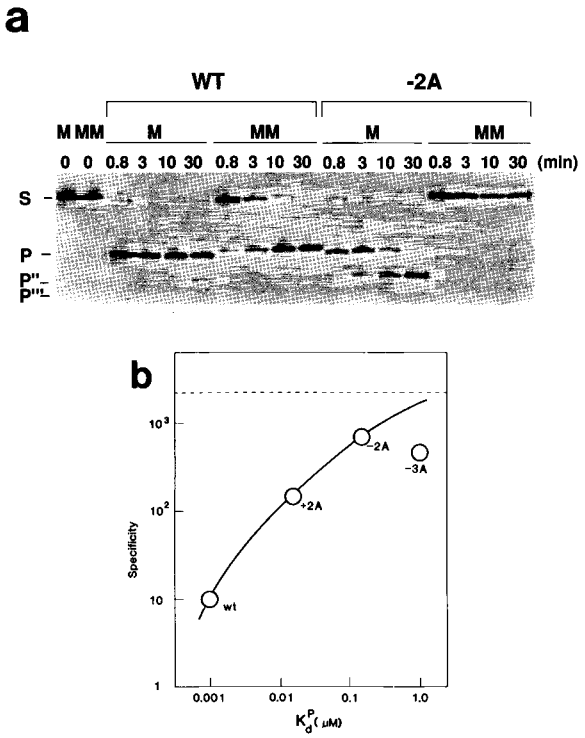


Figure 6. Mutant Ribozymes Show Increased Specificity, Decreased Fidelity

(a) wt and −2A mutant ribozymes (10 nM) were incubated with ~1 nM 5′ end–labeled matched RNA substrate (M) or a mismatched substrate (MM) and 2.0 mM G (i.e., "$(k_{cat}/K_m)^S$ conditions"). Comparisons under these conditions are appropriate for evaluating specificity (Figure 5a). In all cases the primary product was P ($G_2CCC_5^6CU$), produced by G cleavage at the phosphate corresponding to the 5′ splice site in the pre-rRNA. The −2A mutant ribozyme gave increased amounts of products 2 nt shorter (P″, $G_2CCC_5^6$) and 3 nt shorter (P‴, G_2CCC). P produced from S_M and that produced from S_{MM} have slightly different electrophoretic mobilities because of the single nucleotide sequence difference; an analogous situation occurs for P″. The +2A mutant cleaved both substrates to give primarily P and P″, while the −3A mutant gave P, P″, and a shorter product (presumably P‴) with S_{MM} and P, P′ (G_2CCCUC), and P″ with S_M (data not shown). Mapping of these cleavage sites is shown elsewhere (D. H., manuscript submitted). Additional products arise both from miscutting of bound S and from recutting of the primary product P at new sites (Young, 1990; D. H., manuscript submitted).

(b) Specificity increases with weaker RNA binding. Specificity as defined in Figure 5a was determined from rate measurements with 10 nM wt, +2A, or −2A ribozyme or 40 nM −3A ribozyme, 400 μM GTP, and ~1 nM S_M or S_{MM}. Specificity is affected by the concentration of G, increasing as [G] decreases for the wt ribozyme (Herschlag and Cech, 1990b). The concentration of GTP used here is not saturating, so increasing it would be expected to decrease the observed specificity.

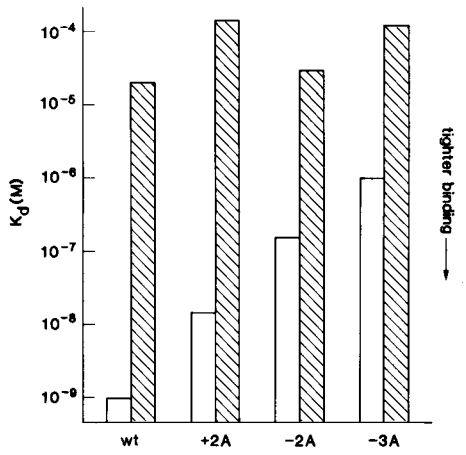


Figure 7. Comparison of RNA- and DNA-Binding Properties of Mutant Ribozymes

Equilibrium dissociation constants for the reaction product, ribo-(GGCCCUCU), or deoxyribo-(CCCUCU) were determined by competitive inhibition as described in the Experimental Procedures. Open bars, RNA product; shaded bars, DNA product. The two Gs at the 5′ end of the ribonucleotide product do not base-pair with the 5′ exon-binding site, and their small effect on binding (Herschlag and Cech, 1990b) is negligible for the considerations here.

Differential Effect on DNA versus RNA Binding

The ability of the variant ribozymes to bind the all-deoxy product (dP), d(CCCUCU), was tested by determination of K_I^{dP}. The −2A ribozyme binds DNA almost as well as does wt, while the other mutants show 6- to 7-fold destabilization (Figure 7 and Table 1). In contrast to the modest effect of the −2A mutation on DNA binding, RNA binding is reduced by 150-fold. Similarly, the −3A mutation decreased binding of RNA 200-fold more than that of DNA. This mutational result provides strong independent evidence for the model that DNA binds mostly by base-pairing, whereas RNA binds by base-pairing plus tertiary interactions involving its 2′-OH groups (Sugimoto et al., 1989a, 1989b; Herschlag and Cech, 1990b, 1990c; Pyle et al., 1990; Pyle and Cech, 1991). In addition, it is now apparent that J1/2 is important in allowing or promoting the tertiary interactions between specific substrate 2′-OH groups and the ribozyme.

Small Effect on Chemical Step

To evaluate the integrity of the catalytic center of the ribozyme, $(k_{cat}/K_m)^G$ was measured in single-turnover reactions at high [E] by varying the concentration of G. $(k_{cat}/K_m)^G$ is the second-order rate constant for the reaction of E^S and G. For the wt ribozyme, this rate constant is limited by the actual chemical step rather than the binding of G (Herschlag et al., 1991). The mutant ribozymes had reduced values of $(k_{cat}/K_m)^G$, but all were within an order of magnitude of that of the wt ribozyme (Table 1).

Guanosine binding was estimated to be weakened 3- to 5-fold in the mutant ribozymes (Table 1). The estimate of K_d^G and the measured value of $(k_{cat}/K_m)^G$ were used to estimate the rate constant for the chemical step of $k_c \approx$ 300–400 min^{-1} for the mutants, which is only ~2-fold slower than that for the wt (Table 1).

To test whether $(k_{cat}/K_m)^G$ still represented the actual chemical reaction for the mutant ribozymes, as with the wt ribozyme, experiments with phosphorothioate substrates were performed. Substrates with a [R_P]-phosphorothioate substituted for the phosphate at the cleavage site are readily cleaved by the wt ribozyme (McSwiggen and Cech, 1989). Under conditions of low [G] in which the chemical step is rate limiting, ribozyme-catalyzed cleavage with guanosine shows a "thio effect" ($k_{phosphate}/k_{phosphorothioate}$) of

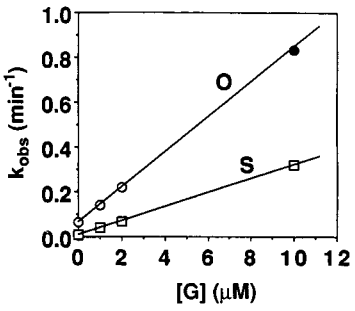


Figure 8. The Chemical Step Limits the Rate of the +2A Ribozyme at Low G Concentration, as with the wt Ribozyme

$(k_{cat}/K_m)^G$ was measured as described in the text, with saturating concentrations of the +2A ribozyme (~100 nM) and either normal phosphate-containing RNA substrate (open circles, closed circle) or RNA substrate with a single R_P–phosphorothioate substituted at the reaction site (open boxes). The closed circle datum point was subjected to a small correction of <10% as described by Herschlag et al. (1991). Slope = $(k_{cat}/K_m)^G$. Ratio of slopes = 7.6×10^4 M^{-1} min^{-1}/3.3×10^4 M^{-1} min^{-1} = 2.3, as expected if the chemical step (the actual attack of guanosine on the RNA) was rate limiting under these conditions. Y-intercept = k_c (–G). The ratio of intercepts of 8 ± 2 is similar to that found for the wt ribozyme, indicative of a rate-limiting chemical step in the hydrolysis of S (Herschlag et al., 1991).

Table 2. Cleavage of Thio-Substituted RNA to Identify the Chemical Step in Reactions Catalyzed by the Wild-Type and Mutant Ribozymes

Ribozyme	Thio effect[a] on $(k_{cat}/K_m)^S$[b]	Thio effect[a] on $(k_{cat}/K_m)^G$[c]
wt	1.06 (±0.1)[d]	2.3 (±0.3)[d]
+2A	1.0 (±0.1)	2.3 (±0.4)[e]
–2A	1.1 (±0.1)	2.0 (±0.5)[f,g]
–3A	1.05 (±0.1)	1.7 (±0.4)[g,h]
		2.0[i]

[a] Thio effect in the ratio of rate constants for reaction of the phosphate-containing substrate and the corresponding substrate containing a phosphorothioate (P-S) at the cleavage site: thio effect = k(p*GGCCCUCUAGU)/k([R_P]p*GGCCCUCU(P-S)AGU). Reactions were carried out with 50 mM MES (pH 6.7) and 10 mM $MgCl_2$ at 50°C. Values in parentheses are estimated limits of uncertainty.

[b] This is the rate constant for the reaction: $E_G + S^* \rightarrow P^*$, determined with 5 nM +2A or –2A ribozyme or 40 nM –3A ribozyme, 800 μM G and ~1 nM S* (phosphate or phosphorothioate). This concentration of G is saturating, as it is much larger than K_m^G, even though it is not significantly greater than K_d^G (Herschlag and Cech, 1990b).

[c] This is the rate constant for the reaction: $E^{S^*} + G \rightarrow P^*$. Conditions are given in the individual footnotes.

[d] From Herschlag et al. (1991).

[e] Determined from the data in Figure 8.

[f] Determined from data analogous to that shown for the +2A ribozyme in Figure 8 (100 nM –2A ribozyme, 0–10 μM G).

[g] $(k_{cat}/K_m)^{G,app}$ was determined, since the concentration of E was not high enough to give complete binding of S*. The thio effect is expected to be the same as that for $(k_{cat}/K_m)^G$ as follows. With sufficiently low G, the chemical step (or a step involved in binding of G) must become rate limiting, rather than binding of S. Furthermore, it has been shown with the wt ribozyme that thio substitution does not affect binding of S. Thus, the lack of saturation is not expected to affect the thio effect under these conditions, so that the thio effect on $(k_{cat}/K_m)^{G,app}$ involves only the steps subsequent to binding of S, as is the case for $(k_{cat}/K_m)^G$. Indeed, the larger thio effect on $(k_{cat}/K_m)^{G,app}$ than on $(k_{cat}/K_m)^S$ shows that the concentration of G is sufficiently low to change the rate-limiting step (see Herschlag et al., 1991).

[h] Determined with 40 or 600 nM –3A ribozyme and 0–10 μM G. The same thio effect was obtained with 40 nM ribozyme and 800 μM G at pH 5.2. Lowering the pH slows the chemical step, so that the chemical step instead of binding can become rate limiting (D.H., unpublished data).

[i] Determined by comparison of the ratios of products from the phosphate- and phosphorothioate-containing substrates (D.H., manuscript submitted).

2.3, whereas under conditions in which substrate binding or product release is rate limiting, the thio effect is 1 (Herschlag et al., 1991). For the +2A mutant ribozyme, the thio effect on $(k_{cat}/K_m)^G$ was 2.3 (Figure 8), the same as that of the wt. Similar thio effects were obtained for the other mutant ribozymes, as summarized in Table 2. This suggests that the same step, chemical reaction, is largely or entirely rate limiting for $(k_{cat}/K_m)^G$ for the mutant and wt ribozymes. In contrast to the significant thio effect on $(k_{cat}/K_m)^G$, there was no thio effect on $(k_{cat}/K_m)^S$ for any of the mutant ribozymes (Table 2). This result shows that the chemical step is not rate limiting under these conditions of subsaturating S and saturating G and is consistent with binding of the RNA substrate being rate limiting for $(k_{cat}/K_m)^S$, as described above.

In the absence of guanosine, the ribozyme catalyzes a slower hydrolysis of the RNA substrate at the same phosphodiester linkage normally cleaved by G (Herschlag and Cech, 1990a). The rate constant for hydrolysis of S bound to the ribozyme is k_c(–G). The mutant ribozymes have values of k_c(–G) 2- to 6-fold lower than that of the wt enzyme (Table 1). Thus, as in the case of the G-dependent reaction, there is some perturbation in the chemical step. Because the effects are small, we suggest that the mutations introduce a small structural defect, altering slightly the orientation of the bound substrate relative to the catalytic core of the ribozyme and therefore making it harder to achieve the required transition state geometry.

What Slows Reactions of the –3A Ribozyme?

The reaction of E_G with S, represented by the second-order rate constant $(k_{cat}/K_m)^S$, is ~20-fold slower for the -3A ribozyme than for the other ribozymes (Table 1). The other ribozymes are limited by binding of S, so it initially seemed reasonable that the lower value of $(k_{cat}/K_m)^S$ for the –3A ribozyme might indicate that the chemical step had become rate limiting. However, the absence of a thio effect on $(k_{cat}/K_m)^S$ (Table 2), contrasted with the thio effect of 2 on the chemical step, shows that the chemical step is not rate limiting for $(k_{cat}/K_m)^S$. A pulse–chase experiment with the –3A ribozyme, as described for +2A and –2A above, revealed that at least 30% of the S* in the $E_G^{S^*}$ ternary complex reacts to give P* instead of dissociating (data not shown). This means that the barrier for dissociation of S is similar to or larger than the barrier for the chemical step, the situation shown in Figure 4c. Thus, in the forward reaction, which entails binding of S followed by the chemical step, some step associated with binding is at least partially rate limiting.

The question then arises: why is the apparent rate of binding of S (k_{on}^{app}) for the –3A ribozyme ~20-fold lower

than k_{on}^{S} for the other ribozymes (Table 1)? This low value of k_{on}^{app} can be explained by an additional step involved in binding, as depicted in equation 1, such that $k_{on}^{app} = k_{on}^{S} \times K_1$, with $k_{on}^{S} = 10^8\ M^{-1}\ min^{-1}$ for binding to the active (E_a) conformer, the same value as for the other ribozymes, and $K_1 = [E_a]/[E_i] = 1/20$, so that most of the ribozyme is in the inactive (E_i) conformation.

$$E_i \overset{K_1}{\rightleftharpoons} E_a \underset{k_{off}^{S}}{\overset{k_{on}^{S}[S]}{\rightleftharpoons}} E_a^S \quad (1)$$

Alternatively, the ~20-fold lower value of k_{on}^{app} for the −3A ribozyme could arise if only 1/20th of the ribozyme were active and the rest "dead," as depicted in equation 2. (By "dead" it is meant that this E_i is not in equilibrium with the active E_a on the time scale of these experiments.)

$$E_i \not\rightleftharpoons E_a \underset{k_{off}^{S}}{\overset{k_{on}^{S}[S]}{\rightleftharpoons}} E_a^S \quad (2)$$

The models of equations 1 and 2 can be distinguished by comparing the values of K_d^P or K_d^S observed with ribozyme in excess versus oligonucleotide in excess. According to model 2, E_i never binds oligonucleotide, so it behaves as if it were simply not present; K_d^{obs} will therefore be larger with E in excess than with oligonucleotide in excess. In model 1, on the other hand, E_i and E_a interconvert, so they act as if they were a single species, and K_d^{obs} is the same with E in excess as with oligonucleotide in excess. Pulse–chase experiments with 1 μM and 2.6 μM ribozyme gave 20% and 30%, respectively, of S* trapped as P* (data not shown). This provides a lower limit for the fraction of S* bound with these ribozyme concentrations, and thus an upper limit of $K_d^{obs} \leq 4$ μM with ribozyme in excess. This value is similar to $K_d^P = 1$ μM measured with oligonucleotide in excess (Table 1), and not ~20-fold higher, as would be expected if a large fraction of the ribozyme were dead. (Note that S and P bind similarly to the wt and −2A ribozymes [Herschlag and Cech, 1990a; Pyle et al., 1990], so it seems reasonable to compare K_d^S and K_d^P values for the −3A ribozyme.) A modified version of model 2 in which E_i binds oligonucleotides with the same affinity as E_a is also discounted by the pulse–chase experiment, since only 1/20th of the E^{S*} could form P* in that case. This is significantly less than the 20% trapping seen with 1 μM ribozyme or the 30% trapping seen with 2.6 μM ribozyme. Thus, we conclude that the low value of $(k_{cat}/K_m)^S$ for the −3A ribozyme is explained by model 1 and not by a preponderance of dead ribozyme. The possibility of a small amount of dead −3A ribozyme cannot be eliminated, but it does not appear to account for the bulk of the rate effect.

Discussion

The individual steps required for cleavage of RNA by the Tetrahymena ribozyme have recently been kinetically isolated and their rates determined (Herschlag and Cech, 1990a, 1990b). This study represents application of this kinetic framework to understanding the effects of mutations of the ribozyme.

It is initially surprising to observe the mutant phenotype varying from mild to strong and even from positive to negative depending on the concentrations of the ribozyme and its substrates used to assess activity (Table 3). At low RNA substrate and saturating guanosine concentrations, the +2A and −2A mutants are indistinguishable from the wt. With saturating RNA substrate and low guanosine, the mutants appear moderately defective. But when both substrates are saturating, the reaction rate increases greatly; the increase approaches two orders of magnitude for the −2A ribozyme. Superficially, this changing phenotype might seem inconsistent, but it simply results from the three observed rates being dominated by three very different elemental processes, as summarized in Table 3.

The mutant phenotype also varies from mild to severe depending on which substrate is studied. Although the mutations do not significantly alter the rate constant $(k_{cat}/K_m)^S$ for the matched RNA substrate, $(k_{cat}/K_m)^S$ for a mismatched substrate is greatly decreased as the rate-limiting step changes from substrate binding to the chemical step. Had the mutations been evaluated only under these $(k_{cat}/K_m)^S$ conditions (subsaturating RNA substrate), they would have appeared to have no phenotype or a severe down phenotype depending on which substrate was chosen for study.

Weak Binding of RNA and Fast Turnover

The wt ribozyme binds its RNA substrate and product extremely tightly (K_d = 1–2 nM, Herschlag and Cech, 1990a; see also Pyle et al., 1990). The RNA product is analogous to the 5′ exon in self-splicing, and presumably it interacts with the intron core in the same manner. The tight binding is thought to serve an indispensable biological function during RNA splicing, being necessary for the intron to retain the cleaved 5′ exon long enough to accomplish the exon ligation step (Herschlag and Cech, 1990b; see also Danenberg et al., 1989). However, tight binding greatly

Table 3. Strong Dependence of Phenotype of J1/2 Mutations on Substrate Concentrations Can Be Understood in Terms of Changes in Rate-Limiting Step

Substrate Concentrations	Corresponding Kinetic Parameter	Phenotype of J1/2 Mutations[a]	Rate-Limiting Step
Saturating [G], low [S]	$(k_{cat}/K_m)^S$	0	RNA binding
Low [G], saturating [S]	$(k_{cat}/K_m)^G$	−	Chemical cleavage
Saturating [G] and [S]	k_{cat}	++	Product release

[a] 0, no effect (<2-fold); −, as much as 7-fold down; ++, as much as 60-fold up.

limits the ribozyme's activity as an enzyme, where RNA substrates are cleaved with multiple turnover. The ribozyme binds its 5′ cleavage product P so tightly that it is easily saturated. The mutations analyzed here alleviate this "problem" by weakening the E^P interaction, thereby facilitating product dissociation and increasing k_{cat} by as much as 60-fold. This increase occurs without a measurable decrease in $(k_{cat}/K_m)^S$ for the −2A and +2A mutants. Because k_{cat} determines the rate of cleavage with saturating S and $(k_{cat}/K_m)^S$ determines the rate with subsaturating S, these mutant ribozymes have cleavage activity greater than or equal to that of the wt ribozyme at all concentrations of E and S, as long as G is saturating.

Enhancement of catalytic turnover by weakening the binding of a reaction product should be possible in any system that is rate limited by product release. The increased turnover number of deletion mutants of the RNA subunit of RNAase P is explicable on this basis (Waugh et al., 1989). Similarly, limited proteolysis of the protein enzyme carboxypeptidase A increases the k_{cat} for ester hydrolysis 10-fold, which has been attributed to facilitation of a rate-limiting product dissociation step (Solomon et al., 1990).

Degrees of Catalytic Perfection

Turning now to conditions in which the RNA substrate is not saturating, cleavage by the wt ribozyme has been found to be limited by a diffusive step, the binding of the RNA substrate (Herschlag and Cech, 1990a). The free energy barrier for the chemical step is lower than that for substrate binding, so the chemical step does not affect the rate observed under these $(k_{cat}/K_m)^S$ conditions (Figure 4c). Thus, an improvement in transition-state stabilization would make no impact on the overall rate of cleavage, and there would be no basis for selection of such improvement during evolution. By this limited criterion, the catalytic center of the ribozyme is "perfect" (Albery and Knowles, 1976, as discussed by Herschlag and Cech, 1990a). A more complete definition of catalytic perfection would include additional properties, such as high turnover number (discussed above), high specificity, and high fidelity, as summarized in Table 4.

Specificity is the cleavage of matched relative to mismatched RNA, evaluated under $(k_{cat}/K_m)^S$ conditions. The wt ribozyme has modest specificity, at least under our standard reaction conditions. Because the chemical step occurs faster than substrate dissociation, this ribozyme cleaves essentially every RNA molecule, matched or mismatched, that binds. In contrast, weaker binding of RNA puts the mutant ribozymes into a realm in which they can take advantage of the large difference in K_d between the matched and mismatched substrates (Figure 5b). Specificity approaches the calculated maximum value in the case of the −2A ribozyme (Figure 6b). Lowering the guanosine concentration likewise gives a large increase in specificity, in that case by slowing the chemical step to allow equilibration of substrate binding prior to cleavage (Herschlag and Cech, 1990b).

The principle that excessively tight binding decreases discrimination has implications for the use of antisense nucleic acids or ribozymes for the targeted destruction of RNA in vivo. Making binding tighter, such as by lengthening the region of base-pairing, does not always increase specificity and in fact can decrease specificity (Herschlag, 1991). Our study of the J1/2 mutants illustrates that, in the case of ribozymes, specificity can be manipulated or tuned through engineering. There have been several attempts to reengineer the specificity of protein enzymes, some of which have been quite successful (see references in Bonneau et al., 1991).

The J1/2 mutations also lead to infidelity: substantial cleavage of the RNA at positions other than the correct internucleotidyl linkage. Additional characterization has revealed that the P1 helix of the mutants can dock into the tertiary interactions in different registers, promoting cleavage at incorrect sites (D. H., manuscript submitted). Thus, while these ribozymes are very good at recognizing their cognate RNA substrate, they are less accurate in positioning the site of cleavage of the properly chosen molecule. They have high specificity, but limited fidelity. For many applications involving targeted cleavage of RNA using a ribozyme in trans, specificity is clearly much more important than fidelity. It is critical that the correct target is attacked, but not important how it is inactivated. However, for the cis reaction, RNA splicing, fidelity is of prime

Table 4. Some Qualities of a "Perfect" Catalyst

Quality	wt	+2A and −2A Mutants
Rate limited by diffusive step[a]	+	+
High turnover number[b]	−	+
High specificity[c]	−	+
High fidelity[d]	+	−

[a] Kinetic parameter being assessed is $(k_{cat}/K_m)^S$, which for "perfection" must equal k_{on}^S, which must in turn be limited by a diffusive step. For an RNA enzyme, the rate of formation of a double helix between two complementary oligonucleotides is taken as the upper limit in evaluating perfection, even though it is slower than the rate of diffusional encounter (see Herschlag and Cech [1990a] for a more complete discussion).

[b] Kinetic parameter being assessed is k_{cat}. While it is common to consider high turnover at physiological substrate concentration to be a hallmark of catalytic perfection (Fersht, 1974; Jencks, 1975), the concept of a physiological substrate concentration is not readily applied to an artificial enzyme like the L-21 ScaI ribozyme. Nevertheless, the main point here is that the mutant ribozymes are less easily saturated than the wild type, so they give better or equal cleavage at any [S], as long as G is saturating.

[c] Specificity is defined as the rate of cleavage of the matched RNA substrate relative to that of a mismatched RNA substrate when both are present at the same concentration, competing for the same ribozyme (Figure 5a). The J1/2 mutants are designated "+" because they approach the maximum specificity obtainable from the difference in energy of base-pair formation between matched and mismatched substrates. A ribozyme that discriminated against mismatched substrates by additional mechanisms such as steric hindrance could in principle attain still higher specificity.

[d] Fidelity is defined as the rate of cleavage of the correct UpA linkage, which corresponds to the 5′ splice site in the pre-rRNA, relative to the rate of cleavage of all sites on the bound substrate RNA. The fidelity of the wt ribozyme approaches unity. The infidelity of the mutant ribozymes is analyzed in greater detail elsewhere (D.H., manuscript submitted).

importance; infidelity in the guanosine-addition step of self-splicing can still allow exon ligation to proceed, resulting in deletion or insertion of nucleotides in the ligated exons (Waring et al., 1986; Barfod and Cech, 1989). Thus, J1/2 may serve a critical biological function in Tetrahymena pre-rRNA splicing, promoting high fidelity in the choice of the 5′ splice site.

Energetic Implications

How do the mutations destabilize the ribozyme–RNA interaction? With the wt ribozyme, two components contribute to the stability of the E^S and E^P complexes: First, base-pairing between the RNA or DNA oligonucleotide and its binding site on the ribozyme forms the P1 duplex. Second, for RNA only, tertiary interactions increase the equilibrium association constant some 10,000-fold beyond that attributable to base-pairing at 50°C in 10 mM $MgCl_2$ (Herschlag and Cech, 1990a, 1990b; see also Sugimoto et al., 1988, 1989a, 1989b; Pyle et al., 1990). To a large extent the increased binding of RNA relative to DNA is localized to two of the 2′-hydroxyl groups (Pyle and Cech, 1991). Now considering the mutant ribozymes, −2A binds RNA with a much reduced affinity, but binds DNA almost as well as does the wt ribozyme (Figure 7). The simplest interpretation is that the mutation does not affect the base-pairing component of binding, but does decrease the ribozyme's ability to derive full advantage from the tertiary interactions, which are RNA specific. The +2A mutation perturbs DNA binding almost as much as RNA binding, perhaps indicating that in this mutant there are negative interactions that actually interfere with base-pairing; alternatively, even DNA might have some small additional interaction beyond base-pairing that is disrupted by the +2A but not by the −2A mutation.

The following simple model could account for the weakened interactions of the mutant ribozymes with RNA. Altering the length of J1/2 makes it more difficult for the P1 helix to dock into its binding site in the catalytic core of the ribozyme, the interaction that involves the RNA 2′-hydroxyls. P1 is still able to dock, but only at the energetic expense of some distortion of the ribozyme (e.g., partial unzipping of P2 to replace the missing J1/2 nucleotides). In the case of the −2A mutant, the binding energy decreases by 150-fold (0.001 to 0.15 μM, Table 1), but is still 70-fold stronger than calculated for base-pairing alone (10 μM; Freier et al., 1986, as described by Herschlag and Cech, 1990b). In free energy terms, the −2A mutant has lost 3.2 kcal/mol of the 6.0 kcal/mol extra binding energy observed with the wt ribozyme. Thus, about 3 kcal/mol of the extra binding energy disappears, presumably used to compensate for the structural distortion now required for P1 to enter its binding site. Once P1 binds, its position relative to the catalytic center is almost normal, as judged by k_c and $k_c(-G)$ being only moderately decreased. An alternative model, in which J1/2 directly provides the tertiary interactions with P1, has been tested and found not to hold (D. H., manuscript submitted).

Structural Implications: J1/2 Is Nonconserved but Important

J1/2 is not phylogenetically conserved in length or sequence in group I introns. Of 56 group I introns that contain a P2 helix, 38 have a J1/2 of 0 or 1 nucleotides (nt), 13 have a J1/2 of 2 or 3 nt, and 5 have a J1/2 $\geq$ 5 nt (Michel and Westhof, 1990). Based on this lack of conservation, we originally thought we would be able to delete J1/2 without major consequence. Instead, we find that the three nucleotides in J1/2 are required for the substrate-containing helix, P1, to be positioned optimally with respect to the tertiary contacts with its 2′-OH groups and with respect to the catalytic apparatus. We have not examined the nucleotide sequence requirements of J1/2, only the length requirement.

Twenty-eight group I introns, not including the Tetrahymena intron, have a tetranucleotide L2 terminal loop with sequence GNRA. Michel and Westhof (1990) have noted that the common G in P1 (the G that base-pairs with the splice site U) is separated from this GNRA by a constant 12 nt, counting along P2, across J1/2, and along P1. This leads to a model whereby P1, the 0–3 bases of J1/2, and P2 stack coaxially as a helical rod, and a specific interaction with the GNRA at the end of P2 helps position the U·G pair for cleavage. For the Tetrahymena intron, our data indicate that J1/2 is involved in this process of positioning the correct U·G pair at the cleavage site. The length of P1 is also important for positioning (Doudna et al., 1989), as are the two cross-linkable nucleotides on either side of P2.1 (W. Downs and T. R. C., unpublished data). However, J1/2 does not appear to act as a rigid continuation of P1: we find that lengthening and shortening of J1/2 have similar rather than opposite effects, both increasing the use of reaction sites preceding the normal site. Therefore, it seems quite likely that the J1/2 nucleotides of the Tetrahymena ribozyme engage in some specific interaction with the core.

It thus appears that in different group I introns, J1/2 contributes to positioning the reaction site in at least two different ways. In the 28 introns that follow the "rule of 12," J1/2 may simply continue a helical rod as proposed by Michel and Westhof (1990), whereas in the Tetrahymena and presumably other group I introns, it may engage in more specific interactions.

Concluding Remarks

We have seen that an RNA catalyst that is well adapted to its biological function, RNA self-splicing, has severe limitations as a multiple-turnover endoribonuclease, a role for which it was not selected during evolution. Small changes in the length of the J1/2 region give a dramatic enhancement of both turnover number and specificity, thereby "correcting" the intron's limitations as an enzyme. One might have expected that engineering such properties would be exceedingly difficult, perhaps requiring sophisticated information about ribozyme tertiary structure. Quite to the contrary, there may be nothing very special about the J1/2 mutations. Based on our understanding of the kinetic basis of the phenotype, we expect that a variety of alterations that destabilize interactions between the P1 helix and the ribozyme core will have a similar effect. It remains to be seen whether other mutations will, like the J1/2 mutations, act locally to destabilize the RNA–RNA interaction, or whether they will introduce more global

damage, affecting guanosine binding, the catalytic apparatus, or RNA folding.

Experimental Procedures

Materials

Unlabeled nucleoside triphosphates were purchased from P-L Biochemicals; [γ-^{32}P]ATP was purchased from ICN or New England Nuclear. T4 polynucleotide kinase, DNA ligase, and DNA polymerase were from U. S. Biochemical Corporation, calf intestinal phosphatase from New England Nuclear, and ScaI endonuclease from New England BioLabs.

Plasmid Construction

Parent plasmids pTZIVS+ and pT7L-21f1 are derivatives of pBGST7 (Been and Cech, 1986) and pT7L-21 (Zaug et al., 1988), respectively, that contain a bacteriophage f1 origin of replication. Oligonucleotide-directed phagemid mutagenesis of parent plasmids was performed as described by Kunkel et al. (1987). Plasmid pT7J1/2+2A, encoding the +2A variant ribozyme, was derived by mutagenesis of pTZIVS+. All other variants were derived from pT7L-21f1. The sequence of the variant plasmids was verified by chain termination primer extension sequencing using reverse transcriptase as described by Zaug et al. (1984). In all plasmids, a promoter for phage T7 RNA polymerase is positioned such that transcription begins with nucleotide 22 of the intron.

Ribozyme Preparation

Plasmid DNA was linearized with ScaI restriction endonuclease, such that transcription terminated at nucleotide 409, 5 nt preceding the natural 3′ end of the intron. Transcription conditions were described previously (Zaug et al., 1988). Transcription products were separated by 4% polyacrylamide denaturing gel electrophoresis. Following visualization by brief UV shadowing, the appropriate band was excised, eluted by the crush-and-soak procedure, and twice ethanol precipitated. The 5′ terminal sequence of the ribozyme RNA, including the J1/2 region, was verified by chain termination primer extension using reverse transcriptase as previously described (Zaug et al., 1984).

Oligonucleotide Preparation

RNA substrates and products were synthesized using phage T7 RNA polymerase and synthetic DNA templates (Milligan et al., 1987) as described previously (Zaug et al., 1988). Following treatment with calf intestinal phosphatase, RNA was 5′ end-labeled using [γ-^{32}P]ATP (equimolar to the RNA) and T4 polynucleotide kinase and purified by denaturing 20% polyacrylamide gel electrophoresis. The specific activity of the RNA was estimated from the specific activity of the [γ-^{32}P]ATP. The R_P–phosphorothioate substrate was a gift of J. McSwiggen and was prepared as described by McSwiggen and Cech (1989). The DNA product oligonucleotide was prepared using an Applied Biosystems 380B DNA synthesizer. Following deprotection, the DNA was purified by ion exchange chromatography (DEAE fractigel [Supelco], batchwise elution with 1 M tetraethyl ammonium bicarbonate) followed by reverse-phase separation (Sep-pak [Waters], eluted with 50% CH_3CN).

Ribozyme Kinetics

General methods followed those described by Herschlag and Cech (1990a, 1990b), and methods involving the phosphorothioate substrate were according to Herschlag et al. (1991). Ribozyme was preincubated in reaction buffer for 10–20 min at 50°C prior to addition of the RNA substrate to initiate reaction. Extending the preincubation time had less than a 2-fold effect on reaction rates. Reactions were performed in 10 mM $MgCl_2$, 50 mM MES (pH 6.7; pH 7.0 at room temperature, corrected to 50°C according to Good et al., 1966) at 50°C. Portions of the solution were removed at the indicated times and quenched on ice with ~1.5 vol 90% formamide containing 20 mM EDTA, 200 μM Tris (pH 7.5), 0.5% xylene cyanol, 0.5% bromophenol blue. Reaction products were resolved from substrate by electrophoresis in a denaturing 20% polyacrylamide gel, and the fraction product was quantitated with an AMBIS radioanalytic imager. Single-turnover reactions were generally followed for about 3 half-lives and obeyed first-order kinetics, with an end-point ~5% unreactive substrate. Throughout the text, "G" is used as an abbreviation for either guanosine or GTP; these substrates have similar activity as nucleophiles. The form of G that was used in each experiment is specified in the legend to each figure and table.

Specific protocols for the determination of the kinetic parameters listed in Table 1 are given below.

Determination of k_{cat}

Reactions were performed with 10 nM ribozyme and 800 μM guanosine or 500 μM GTP. (The choice of guanosine or GTP did not alter the value.) RNA substrate concentrations were as follows: +2A ribozyme, 0.5–10 μM; −2A, 0.5–16 μM; and −3A, 0.1–16 μM, with at least seven reactions for each ribozyme. Initial rates were determined from the first 25% of reaction. For +2A and −2A ribozymes, increasing the high substrate concentrations gave no increase in observed rate, confirming substrate saturation so that k_{obs} [S]/[E] = k_{cat}. For −3A ribozyme, k_{cat} was determined from a fit to a Michaelis-Menten curve and gave a value within error of that calculated from k_{obs} at the highest substrate concentration.

Determination of K_d^G

Inhibition of single-turnover reactions of 10 nM ribozyme, ~1 nM S*, and 500 μM GTP was measured with 2–4 concentrations of P, as described in Herschlag and Cech (1990a). S* = 5′-^{32}P-labeled S, P = GGCCCUCU. The concentrations of P were: +2A, 10–100 nM; −2A, 150–1000 nM; −3A, 450–3000 nM. For the −2A and +2A mutants, weak binding of P was also confirmed by direct measurement of K_d^G by native gel electrophoresis (Pyle et al., 1990; A. M. Pyle and T. R. C., unpublished data).

Determination of K_d^{dP}

Inhibition of single-turnover reactions of 10 nM +2A or −2A ribozyme or 40 nM −3A ribozyme with ~1 nM S* and 1 mM GTP was measured with 0, 70, and 180 μM dP (d(CCCUCU)), following the procedure outlined in Herschlag and Cech (1990c). The same values were obtained with d(CCCTCT).

Determination of $(k_{cat}/K_m)^S$

Single-turnover reactions were performed with saturating G (800 mM guanosine or 500 μM GTP) and ~1 nM S*. Ribozyme concentrations were: +2A, 5–30 nM; −2A, 5–30 nM; −3A, 30–90 nM. Values of k_{obs} were determined from the slopes of plots of fraction S* versus time. k_{obs} increased linearly with ribozyme concentration, demonstrating subsaturating conditions such that $k_{obs}/[E] = (k_{cat}/K_m)^S$. Values of $(k_{cat}/K_m)^S$ represent the mean of >10 determinations for each ribozyme.

Determination of $(k_{cat}/K_m)^G$

Single-turnover experiments were performed with 200 nM +2A ribozyme, 600 nM −2A ribozyme, or 600 nM −3A ribozyme and 0–10 μM guanosine (more than seven reactions of each ribozyme). The slopes of plots of k_{obs} versus [G] were determined. These values represent the rate constant for the reaction: $E^{S*} + G \rightarrow P^*$. The value obtained with the +2A ribozyme was used directly, as the ribozyme concentration was well over K_d^S = ~15 nM (see following subsection; in addition, saturating +2A ribozyme was confirmed by showing that a 3-fold increase in its concentration gave no significant increase in k_{obs}). The value obtained with the −2A ribozyme was corrected by 1.24, to account for 0.8 saturation with 600 nM ribozyme (from K_d^S = 150 nM; see following subsection). This correction agreed with the small increase of 30% upon increasing the ribozyme concentration to 4.8 μM in a single reaction. In contrast, saturation could not be obtained at attainable concentrations of the −3A ribozyme, so $(k_{cat}/K_m)^{G,app} = 0.3 \times 10^5$ M^{-1} min^{-1} was corrected using K_d^S = 2 μM (see following subsection).

Estimates of the Values of K_d^S

These estimates were used in calculating some of the rate constants. These corrections amounted to <30%, except in the case of the −3A ribozyme (where corrected values are preceded by "~" in Table 1). The dissociation constants of S and P are similar for the wt ribozyme (K_d^S = 2 nM, K_d^P = 1 nM; 50°C, Herschlag and Cech, 1990a) and for the −2A ribozyme (K_d^S = 73 nM and K_d^P = 90 nM; 42°C, Pyle et al., 1990). (The similarity of K_d^S and K_d^P and the value of K_d^P = 150 nM for the −2A ribozyme (50°C) lead to an estimated value of K_d^S = 150 nM and to the value of k_{off}^S in Figure 2b). For the −3A ribozyme, a limiting value of $K_d^S \leq 4$ μM has been estimated (see text). This estimate, the value of K_d^P = 1 μM, and the generality that the values of K_d^S and K_d^P are similar lead to the rough estimate of K_d^S = 2 μM that is used in the calculations herein. Similarly, for the +2A ribozyme, K_d^S is presumed to be about equal to the K_d^P of 15 nM.

Determination of K_d^S

The value for the wt ribozyme is from Herschlag and Cech (1990c),

with the assumption that $K_m^G = K_d^G$ for the DNA cleavage reaction. This assumption is supported by the fact that this reaction is slow, that a similar value is obtained independently in a single-turnover RNA reaction, and that the chemical step is rate limiting in the RNA cleavage reaction under these conditions (see Herschlag et al., 1991). The values for the mutant ribozymes were obtained as follows. Reactions were performed at 30°C and pH 5.2 to increase the binding affinity of G and to slow the reaction (Herschlag et al., 1991). Single-turnover reactions were performed with 50 nM +2A ribozyme, 200 nM −2A ribozyme, and 200 nM −3A ribozyme with 0–1840 μM G (6 concentrations) and ~1 nM S*. (The reaction mixture, without S*, was preincubated at 50°C for 30 min prior to initiating the reactions at 30°C by addition of S*.) Side-by-side comparisons showed that K_m^G was larger for all of the mutant ribozymes than for the wt. ($K_d^G \approx 250$ μM for wt under these conditions; Herschlag et al., 1991; D. H. and P. Legault, unpublished data). Although binding to the mutant ribozymes was too weak to obtain precise values for K_m^G, some saturation was observed, and the side-by-side comparisons suggested that K_m^G is 3- to 5-fold higher for the mutant ribozymes compared with the wt. In Table 1, it is assumed that this ratio is maintained at 50°C, so that $K_m^G = K_d^G \approx 4$ mM.

Determination of k_c(−G)

Single-turnover reactions in the absence of G were performed with ~1 nM S* and ribozyme concentrations of: +2A, 200–600 nM; −2A, 200–500 nM; −3A, 200–700 nM; and they were corrected to saturation as described above for $(k_{cat}/K_m)^G$.

Free Energy Reaction Profiles

The free energy profiles for reaction of S_M (GGCCCUCUA$_6$) and S_{MM} (GGCCCGCUA$_6$) shown in Figure 5b are drawn for $[S_M]$ or $[S_{MM}] = 0.1$ nM, $[G] >> K_d^G$, and [ribozyme] << $[S_M]$ or $[S_{MM}]$ and were calculated from the following rate and equilibrium constants. For the wt ribozyme, the values of K_d^G for S_M and S_{MM} are from Herschlag and Cech (1990a, 1990b), the values of k_{on}^S and k_c are from Figure 2a for S_M, and k_{on}^S for S_{MM} is from Herschlag and Cech (1990b). The value of $k_c = 400$ min^{-1} for S_{MM} used herein is calculated from $(k_{cat}/K_m)^G = 4 \times 10^5$ M^{-1} min^{-1} and $K_d^G = 1$ mM using the equation $k_c = (k_{cat}/K_m)^G \times K_d^G$ (Herschlag and Cech, 1990b, 1990c). This value of k_c is 2-fold higher than that given in Herschlag and Cech (1990b), because $K_d^G = 1$ mM was used in the calculation instead of $K_d^G = 0.5$ mM as used previously. The larger value appears to be a better estimate, although the values are within experimental uncertainty of one another and the differences do not affect any of the conclusions herein or previously reported. For the −2A mutant ribozyme, k_{on}^S and k_c are from Figure 2a for S_M; $K_d^G = 150$ nM is taken from K_d^P (Table 1), because $K_d^G = K_d^P$ for this ribozyme (42°C, Pyle et al., 1990; for the wt ribozyme, the values of $K_d^G = 2$ nM and $K_d^P = 1$ nM are also similar [50°C, Herschlag and Cech, 1990a]). The values of $k_{on}^S = 5 \times 10^7$ M^{-1} min^{-1}, $k_c = 220$ min^{-1}, and $K_d^G = 200$ μM for reaction of S_{MM} with the −2A ribozyme were obtained by assuming the ratio of values for S_{MM}/S_M would be the same as measured for the wt ribozyme. The value of $(k_{cat}/K_m)^S$ calculated for S_{MM} from these values is within 4-fold of the observed value, supporting the validity of this assumption. Equilibrium and rate constants were converted to ΔG values using the equations $\Delta G = -RT\ln K$ and $\Delta G^{\ddagger} = -RT\ln\{hk/k_BT\}$, respectively, in which R is the gas constant, T is temperature in Kelvin (50°C = 323 K herein), h is Planck's constant, and k_B is the Boltzmann constant.

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