Catalysis of RNA Cleavage by the *Tetrahymena thermophila* Ribozyme. 2. Kinetic Description of the Reaction of an RNA Substrate That Forms a Mismatch at the Active Site†

Daniel Herschlag and Thomas R. Cech*

Department of Chemistry and Biochemistry, Howard Hughes Medical Institute, University of Colorado, Boulder, Colorado 80309-0215

Received March 26, 1990; Revised Manuscript Received July 18, 1990

ABSTRACT: The site-specific endonuclease reaction catalyzed by the ribozyme from the *Tetrahymena* pre-rRNA intervening sequence has been characterized with a substrate that forms a "matched" duplex with the 5′ exon binding site of the ribozyme [$G_2CCCUCUA_5 + G \rightleftharpoons G_2CCCUCU + GA_5$ (G = guanosine); Herschlag, D., & Cech, T. R. (1990) *Biochemistry* (preceding paper in this issue)]. The rate-limiting step with saturating substrate is dissociation of the product $G_2CCCUCU$. Here we show that the reaction of the substrate $G_2CCCGCUA_5$, which forms a "mismatched" duplex with the 5′ exon binding site at position −3 from the cleavage site, has a value of k_{cat} that is $\sim 10^2$-fold greater than k_{cat} for the matched substrate (50 °C, 10 mM $MgCl_2$, pH 7). This is explained by the faster dissociation of the mismatched product, $G_2CCCGCU$, than the matched product. With subsaturating oligonucleotide substrate and saturating G, the binding of the oligonucleotide substrate and the chemical step are each partially rate-limiting. The rate constant for the chemical step of the endonuclease reaction and the rate constant for the site-specific hydrolysis reaction, in which solvent replaces G, are each within ~2-fold with the matched and mismatched substrates, despite the $\sim 10^3$-fold weaker binding of the mismatched substrate. This can be described as "uniform binding" of the base at position −3 in the ground state and transition state [Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5631–5640]. Thus, the matched substrate does not use its extra binding energy to preferentially stabilize the transition state. The products $G_2CCCGCU$ and $G_2CUCUCU$ bind the ribozyme less strongly than the matched product, $G_2CCCUCU$. Nevertheless, like the matched product, each binds $\sim 10^4$-fold (~6 kcal/mol) stronger than predicted for base pairing between oligonucleotides. This suggests that disruption of base pairing at position −3 or −5 does not interfere with the tertiary interactions of the ribozyme that are responsible for the enhanced binding stability. The value of $K_m^G = 110\ \mu M$ for the reaction with subsaturating mismatched substrate is less than the dissociation constant of $K_d(E{\cdot}G) \cong 500\ \mu M$ because of a change in rate-limiting step from chemistry at low G to (partially) binding of the oligonucleotide substrate at high G. Rate-limiting chemistry at low G allows enhanced discrimination against the mismatched substrate, without recourse to added denaturants, which have been used previously to enhance specificity. The strong binding of the 5′ exon along with the weak binding of G is proposed to ensure efficient exon ligation during self-splicing.

The intervening sequence (IVS)[1] of nuclear preribosomal RNA from *Tetrahymena thermophila* is excised in the absence of added protein in vitro (Kruger et al., 1982). Catalytic activity is retained in the excised IVS and in shortened forms of this RNA (Cech & Bass, 1986; Zaug & Cech, 1986). A site-specific RNA endonuclease reaction, which is analogous to guanosine addition at the 5′ splice site in the first step of splicing, is catalyzed by a shortened form of the IVS, the L-21 *Sca*I RNA (eq 1; Zaug et al., 1986, 1988). Mutational and phylogenetic data show that the oligonucleotide substrate in this and analogous reactions binds to the 5′ exon binding site, which is a portion of the "internal guide sequence" (Figure 1; Davies et al., 1982; Michel et al., 1982; Waring et al., 1986; Been & Cech, 1986; Zaug et al., 1986; Murphy & Cech, 1989). Throughout this paper we refer to the substrates $G_2CCCNCUA_5$ (N = U, G) as "matched" (N = U) and "mismatched" (N = G) because the *U*-containing substrate forms a Watson–Crick U·A base pair at position −3 with the ribozyme whereas the *G*-containing substrate forms a G·A mismatch at this position (Figure 1).

$$G_2CCCNCUA_5 + G \xrightleftharpoons{\text{L-21 } ScaI} G_2CCCNCU_{OH} + GA_5 \quad (N = U, G) \quad (1)$$

It had initially been expected that a mismatched substrate would react much more slowly than a matched substrate because of weaker binding and perhaps misalignment in the active site. However, such straightforward behavior was not apparent in initial steady-state kinetic investigations. It was found necessary to add urea or formamide to ribozyme cleavage reactions in order to discriminate against the cleavage of mismatched substrates (Zaug et al., 1986). Furthermore, in the absence of denaturant, mismatched substrates were cleaved faster than the matched substrate under saturating (k_{cat}) conditions. A model involving a rate-limiting conformational change was suggested (Zaug et al., 1988).

However, the more refined understanding of the kinetic pathway for reaction of the matched and mismatched substrates from this and the preceding paper (Herschlag & Cech, 1990b) shows that the differential reactivity is understood simply in terms of different binding affinities, without recourse to a hypothetical conformational change. For example, there is little discrimination between the matched and mismatched

† This work was supported by NIH Grant GM 28039 to T.R.C. and by a Fellowship from the Helen Hay Whitney Foundation to D.H. T.R.C. is an Investigator of the Howard Hughes Medical Institute and an American Cancer Society Professor.

* Author to whom correspondence should be addressed.

[1] Abbreviations: IVS, intervening sequence; G, guanosine; MES, 2-(*N*-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; P*, ^{32}P-labeled product; S*, ^{32}P-labeled substrate.

0006-2960/90/0429-10172$02.50/0 © 1990 American Chemical Society

Table I: Summary of Steady-State Kinetic Parameters[a]

substrate	with saturating G: $(k_{cat}/K_m)^S$ (10^7 M^{-1} min^{-1})	k_{cat} (min^{-1})	K_m^S (μM)	with saturating S: $(k_{cat}/K_m)^G$ (10^5 M^{-1} min^{-1})	K_m^G (μM)	hydrolysis reaction: k_{cat}/K_m (10^7 M^{-1} min^{-1})	k_{cat} (min^{-1})	K_m (μM)
$G_2CCCUCUA_5$[b]	9	0.1	0.001	7	–[c]	5	0.1	0.002
$G_2CCCGCUA_5$	2	6	0.3	4[d]	15[e] (110)[f]	0.02	0.4	2

[a] Reactions with 50 mM MES, pH 7, and 10 mM $MgCl_2$ at 50 °C. [b] Data from Herschlag and Cech, preceding paper. [c] No value for K_m^G can be obtained because the addition of G does not increase k_{cat} or k_{cat}/K_m significantly; i.e., the values of k_{cat} and $(k_{cat}/K_m)^S$ are essentially the same for the G-dependent and hydrolysis reactions. [d] Calculated from the rate and equilibrium constants of Scheme I as follows: $(k_{cat}/K_m)^G = k_c/K_d(E{\cdot}G)$ = (200 min^{-1})/(500 μM) = 4×10^5 M^{-1} min^{-1}. The same value is obtained from k_{obsd} at low concentrations of E, S, and G using the equations $k_{obsd} = (k_{cat}/K_m)^G[E{\cdot}S][G]$ and $K_d(E{\cdot}S) = [E][S]/[E{\cdot}S]$ = 2.5 μM (see text). [e] Calculated from $(k_{cat}/K_m)^G$ and k_{cat}. [f] Obtained with subsaturating S; this value is *not* expected to be the same as that with saturating S (see text).

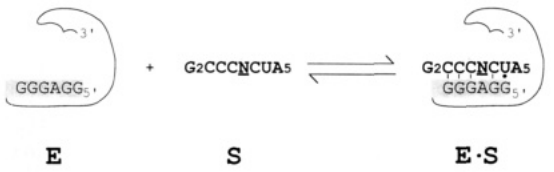


FIGURE 1: Binding of an oligonucleotide substrate (S) to the 5′ exon binding site (shaded) of the L-21 *ScaI* ribozyme (E), which is derived from the *Tetrahymena* intervening sequence (Zaug et al., 1988). N = U for the "matched" substrate and N = G for the "mismatched" substrate.

oligonucleotide substrates with saturating G because the binding of both oligonucleotides is rate-limiting. The mismatch increases the rate of reaction with saturating oligonucleotide substrate because it increases the rate-limiting dissociation of product. Furthermore, the destabilization of equilibrium binding from introduction of the mismatch is that expected for a simple duplex at 50 °C. Thus, perhaps surprisingly, the mismatch does not disrupt the tertiary interactions that provide an additional ~6 kcal/mol of binding energy. In addition, although the mismatched substrate binds to the ribozyme ~10^3-fold less strongly than the matched substrate, the rate constant of the chemical conversion of the ternary complex is essentially the same so that the mismatch does not induce a misalignment that disrupts the transition state. The similar rate of the chemical conversion shows that there is "uniform binding" of the base pair at position –3 in the ground and transition states (Albery & Knowles, 1976) and is consistent with a role for the 5′ exon binding site of holding the substrate at the active site in both states.

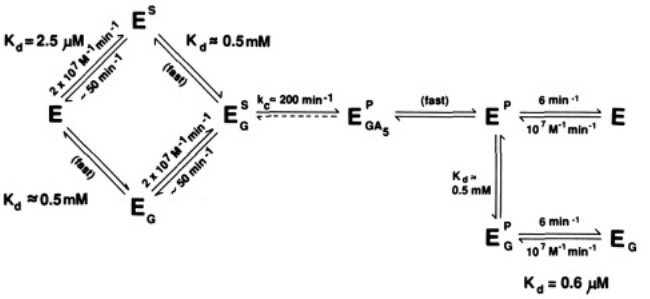
Materials and Methods

Materials. Materials were the same as described in the preceding paper (Herschlag & Cech, 1990b). The oligonucleotides CCCUCU and CUCUCU were made from $pppG_2CCCUCU$ and $pppG_2CUCUCU$, respectively, by treatment with RNase T1, which cleaves 3′ of G residues; they were a gift from J. McSwiggen. The starting oligonucleotides were synthesized by T7 transcription as described previously (Herschlag and Cech, preceding paper; Zaug et al., 1988).

Methods. Kinetic and "pulse–chase" experiments were carried out as described in the preceding paper, at 50 °C with 50 mM MES, pH 7.0 [at 25 °C; pH 6.7 at 50 °C, calculated from Good et al. (1966)], and 10 mM $MgCl_2$. Reactions were followed by PAGE of products from reactions with substrate labeled at its 5′ terminus with ^{32}P (Herschlag and Cech, preceding paper).

Estimation of Errors. There is good precision in data from ribozyme-catalyzed reactions performed side by side, but there is considerably greater variation in independent experiments (Herschlag and Cech, preceding paper). The kinetic constants reported herein suffer from two additional sources of error: (1) Values of Michaelis and dissociation constants have been fit by nonlinear least squares to theoretical binding behavior. These nonlinear least-squares fits to theoretical curves have an additional variable: the "end point" at saturating substrate. This increases the uncertainty of these determinations. (2) A number of the kinetic and equilibrium constants are determined by combining data from different experiments, so that the uncertainty in these values is cumulative. We have not attempted to assign confidence limits to the data; rather, we have tried to estimate the extremes of possible values. This method of error estimation is used because we do not understand the source of all of the variation and because none of the conclusions derived from these data is dependent on a more precise determination of the limits of the uncertainty.

Scheme I



Results

Steady-state, single-turnover, and pulse–chase experiments were carried out for the endonuclease reaction of the mismatched substrate, $G_2CCCGCUA_5$ (eq 1). The results are described in this section and are summarized in Table I and Scheme I. Because of the evidence for random and independent binding of the matched oligonucleotide substrate and of G (Herschlag and Cech, preceding paper), random and independent binding has been assumed for the mismatched substrate and G.

For simplicity, the oligonucleotide substrate is referred to as "S" or "substrate". Guanosine and guanosine triphosphate, either of which can act as the other substrate, are referred to as G and GTP, respectively. The IVS catalyst, the L-21 *ScaI* RNA, is referred to as "E" or the "ribozyme". The oligonucleotide product, $G_2CCCGCU$, is referred to as "product" or "P".

Steady-State Kinetic Parameters and Faster Dissociation of the Mismatched Product Than of the Matched Product. Steady-state kinetics with 7 nM E, 400 μM G, and 0.1–5 μM S gave values of k_{cat}/K_m of 2×10^7 M^{-1} min^{-1} and k_{cat} = 6 min^{-1} for the mismatched substrate. The value of k_{obsd} for formation of product from 2.5 μM S and 400 μM G was linear with [E] from 7–50 nM (data not shown). The value of k_{cat} = 6 ± 2 min^{-1} provides a minimum value for any individual step. In particular, k_{off}^P must be ≥6 min^{-1}. This is much larger than the value of k_{off}^P = 0.1 min^{-1} for the matched product,

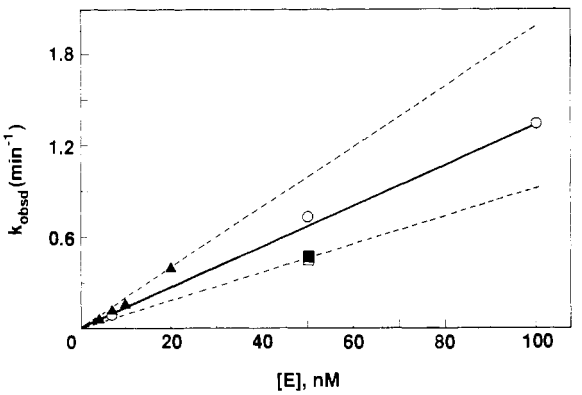
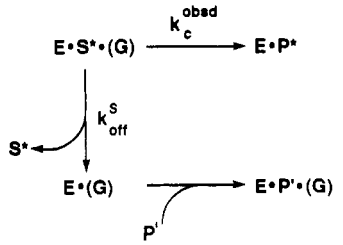


FIGURE 2: Dependence of k_{obsd} on the ribozyme concentration. The endonuclease reaction was carried out with ~1 nM ^{32}P-labeled substrate, p*G_2CCC*G*CUA_5, and 400 or 800 μM G (open and closed symbols, respectively) at 50 °C in 50 mM MES, pH 7, and 10 mM $MgCl_2$. Different symbols represent results from separate experiments. The solid line is the best fit to the data and was used to determine the value of k_{cat}/K_m in the text, and the dashed lines represent the extremes that give the error limits reported in the text.

Scheme II



G_2CCC*U*CU (Herschlag and Cech, preceding paper).

Single-turnover experiments with E in excess of the mismatched substrate provided a second determination of k_{cat}/K_m at saturating G. Figure 2 shows that the value of k_{obsd} increased linearly with the concentration of E over the range of 7–100 nM, with 400–800 μM G. The slope of a plot of k_{obsd} against [E] gives $k_{cat}/K_m = (1.7 \pm 0.7) \times 10^7$ M^{-1} min^{-1}. This value has been corrected to saturating G, using K_m = 110 μM (see below) and the concentration of G; the correction amounted to <20%.

Individual Rate Constants for Binding and Dissociation of G_2CCCGCUA_5 and G_2CCCGCU. Pulse–chase experiments (as outlined in Scheme III of the previous paper) were used to determine the dissociation constant for E·S and the ratio of the rate constants for the chemical conversion and the dissociation of substrate (k_c^{obsd}/k_{off}^S; Scheme II). [It should be noted that the value of k_c^{obsd} (Scheme II) is not necessarily equal to k_c, the rate constant for the chemical conversion of the ternary complex (Scheme I), because the concentration of G present can be less than saturating so that not all of the E·S* complex has G bound. For this reason Scheme II has G placed in parentheses. (The solubility limit of G prevented experiments at significantly higher concentrations.) The observed rate constant of the chemical step in the pulse–chase experiment, k_c^{obsd}, is related to k_c for reaction of the ternary complex according to eq 2, which accounts for the fraction

$$k_c^{obsd} = (k_c)[G]/\{K_d(E{\cdot}G) + [G]\} \quad (2)$$

of E·S* that has G bound. Derivation of this equation assumes that the binding of G is not rate-limiting. Arguments against rate-limiting binding of G are analogous to those presented in the previous paper (Herschlag and Cech, 1990b) and an additional argument is presented below.] In the pulse–chase experiment, ribozyme and labeled substrate (S*) were incubated for 0.3 min, a time sufficient to allow the binding

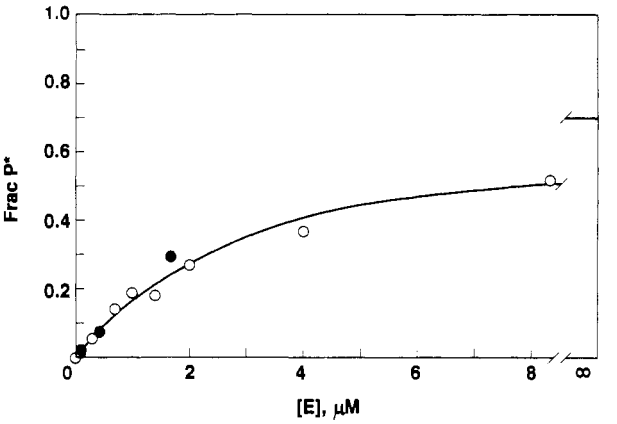


FIGURE 3: Dependence of P* formation on the concentration of ribozyme (E) in a pulse–chase experiment with the substrate, p*G_2CCC*G*CUA_5. Varying concentrations of ribozyme were incubated with S* for 0.33 min followed by a "chase" with 2 μM unlabeled matched P and 800 μM G (○) or 500 μM GTP (●). The fraction of P* trapped that is plotted was corrected from the observed fraction trapped by subtraction of the fraction of S* that formed P* during the preincubation ($fracP^*_{preinc}$ = 0.03–0.13) and renormalization: $fracP^* = (fracP^*_{obsd} - fracP^*_{preinc})/(1 - fracP^*_{preinc})$. The amount of P* formed in these pulse–chase experiments was constant from 0.3 to 2.2 min after the chase. The line is the nonlinear least-squares fit to the data of a binding curve that gives K_d = 3 μM and an end point of $(fracP^*)_{max}$ = 0.7.

equilibrium to be established. At that time, G and excess unlabeled pppG$_2$CCCUCU were added simultaneously. The fraction of S* bound to E could then either react to give P* (k_c^{obsd}) or irreversibly dissociate (k_{off}^S), since the added matched product traps free E with high affinity (K_d = 1 nM; Herschlag and Cech, preceding paper).

The results of such experiments with varying concentrations of E and 800 μM G are shown in Figure 3. The amount of P* formed initially increases with [E] but saturates at high concentrations of E. This concentration dependence gives $K_d = 3 \pm 1$ μM for the E·S* complex formed prior to addition of G and the "chase". The maximum fraction of S* that reacts to form P* in the presence of 800 μM G is 0.7 (Figure 3). This shows that the dissociation of S and the chemical conversion occur at similar rates under these conditions. In contrast, with the matched substrate the chemical conversion is ~10^3-fold faster than dissociation so that all of this substrate is trapped as P* (Herschlag and Cech, preceding paper).

A site-specific hydrolysis reaction, in which solvent replaces G as the nucleophile, was characterized for the matched substrate (Herschlag and Cech, preceding paper). The rate of hydrolysis of the mismatched substrate as a function of the concentration of E in single-turnover reactions exhibited saturation behavior with a value of $K_{1/2} = 2 \pm 1$ μM (data not shown). This is the same as the value of $K_d = 3 \pm 1$ μM determined from the pulse–chase experiments. The value of $K_{1/2}$ obtained from this reaction is expected to represent a true dissociation constant since $k_{off}^S \cong 50$ min^{-1} (see below) is much greater than the value of $k_c(-G) = 0.4 \pm 0.1$ min^{-1}. The value of $k_c(-G)$, the rate constant for the hydrolysis of S* in the binary E·S* complex, was determined from extrapolation of the observed rate constants for the hydrolysis reaction to that for reaction with all of the S* bound to the ribozyme from data with 0.1–8.3 μM ribozyme; the extrapolation was ~15% (data not shown). A value of $K_d = 2.5 \pm 0.5$ μM for the E·S complex, the average of the two determinations above, is used in calculations of individual rate constants below.

The individual rate constants shown in Scheme I for the endonuclease reaction have been determined from the maxi-

mum fraction of S* trapped as product in the pulse–chase experiments; K_d(E·S) = 2.5 μM; and the steady-state rate parameters. The maximum fraction of S* trapped as P* with 800 μM G, $(\text{fracP*})_{max}$ = 0.7, represents the ratio of rate constants in eq 3, which was derived from Scheme II. This

$$(\text{fracP*})_{max} = k_c^{obsd}/(k_c^{obsd} + k_{off}^{S}) \quad (3)$$

fraction for partitioning of the E·S* complex and the value of $(k_{cat}/K_m)^{obsd}$ = (1.4 ± 0.6) × 10^7 M^{-1} min^{-1} with 800 μM G (Figure 2) give k_{on}^{S} = (2 ± 1) × 10^7 M^{-1} min^{-1} with use of eqs 3 and 4. This rate constant for binding is similar to

$$(k_{cat}/K_m)^{obsd} = k_{on}^{S}k_c^{obsd}/(k_c^{obsd} + k_{off}^{S}) = k_{on}^{S}(\text{fracP*})_{max} \quad (4)$$

k_{on}^{S} = (9 ± 3) × 10^7 M^{-1} min^{-1} for the matched substrate (Herschlag and Cech, preceding paper). The slightly smaller rate constant for binding of the mismatched substrate was confirmed in side-by-side determinations of k_{cat}/K_m for the matched and mismatched species (e.g., Figure 6 below). It is consistent with more difficult nucleation of helix formation in the binding of the mismatched substrate (see Herschlag and Cech, preceding paper).

The values of k_{on}^{S} = (2 ± 1) × 10^7 M^{-1} min^{-1} and K_d(E·S) = 2.5 ± 0.5 μM give k_{off}^{S} = 50 min^{-1} (range 20–90 min^{-1}). This value of $k_{off}^{S} \cong 50$ min^{-1} is consistent with the lower limit of 15 min^{-1} that was determined from a pulse–chase experiment in which the unlabeled chase substrate was added 10 s before GTP. In that experiment less than 1% of S* was trapped as product, while controls with the cold substrate and GTP added simultaneously gave 27% of S* trapped as P*.

The observed rate constant for the chemical conversion with 800 μM G (Scheme II) of k_c^{obsd} = 120 min^{-1} (range 50–210 min^{-1}) was determined from $(\text{fracP*})_{max}$ = 0.7 and $k_{off}^{S} \cong 50$ min^{-1}, with use of eq 3.

Substitution of $k_c \geq k_c^{obsd}$ = 120 min^{-1} into the expression for k_{cat} (eq 5) gives $k_{cat} = k_{off}^{P}$ = 6 min^{-1}. This expression

$$k_{cat} = k_c k_{off}^{P}/(k_c + k_{off}^{P}) \quad (5)$$

for k_{cat} assumes that the dissociation of GA_5 is fast. A lower limit of $k_{off}^{GA5} > 1$ min^{-1} was derived for the ternary complex with the matched product (Herschlag and Cech, preceding paper). The following shows that it is likely that this rate constant is much larger than 6 min^{-1} so that the value of k_{cat} for the mismatched reaction represents dissociation of the mismatched product. If a value of k_{off}^{GA5} = 6 min^{-1} is *assumed*, along with a lower limit for $k_{on}^{GA5} = 10^6$ M^{-1} min^{-1}, which is ~10^4-fold below that for diffusion-controlled binding (Eigen & Hammes, 1963), then the dissociation constant for GA_5 would be K_d(E·GA_5) < (6 min^{-1})/(10^6 M^{-1} min^{-1}) = 6 μM. This limit represents ~10^2-fold stronger binding than is observed with G [K_d(E·G) ≅ 500 μM; see below], and binding at closer to the diffusion-controlled rate would give even stronger calculated binding of GA_5 with k_{off}^{GA5} = 6 min^{-1} assumed. However, preliminary experiments suggest that GA does not bind more strongly than G (T. McConnell, D. Herschlag, and T. R. Cech, unpublished results), consistent with earlier more qualitative observations (Been & Cech, 1988). In addition, the pA_5 moiety does not enhance the binding of the oligonucleotide substrate relative to the product, which lacks this moiety. These data suggest that K_d^{GA5} is much larger than 6 μM so that the value of k_{off}^{GA5} is predicted to be much larger than 6 min^{-1}. Thus, k_{cat} = 6 min^{-1} is expected to represent dissociation of the mismatched product. It should be noted that, although there is no evidence for a conformational step that precedes product release and limits the rate, such a step has not been ruled out (see Herschlag and Cech, preceding paper).

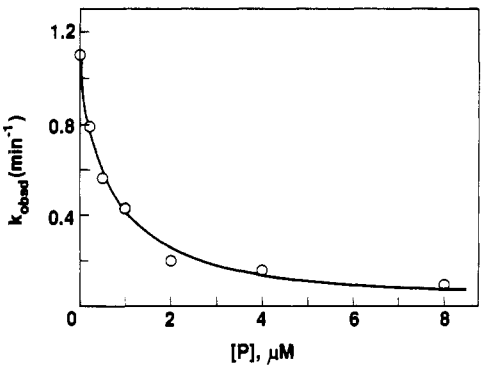


FIGURE 4: Dependence of k_{obsd} on the concentration of the product inhibitor, $pppG_2CCCGCU$. The reaction of ~1 nM $p^*G_2CCCGCUA_5$ and 800 μM G catalyzed by 50 nM ribozyme at 50 °C in 50 mM MES, pH 7, and 10 mM $MgCl_2$. The line is the best fit to the data for K_i = [E][I]/[E·I] = 0.6 μM and competitive inhibition such that the E·I complex is totally inactive.

Scheme III

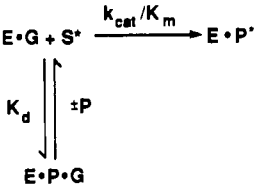


Figure 4 shows inhibition of the endonuclease reaction by the mismatched product, $G_2CCCGCU$. These data give the value of K_i = 0.6 ± 0.2 μM with competitive inhibition. Competitive inhibition means that the observed rate of reaction approaches zero at high concentrations of P and suggests that S and P vie for the same binding site, as expected. Furthermore, the value of K_i from the data of Figure 4 is expected to be equivalent to K_d for E·P as depicted in Scheme III and described as follows. The reactions of Figure 4 were carried out with E in excess over S* (and with saturating G) at a concentration of E that is subsaturating with respect to S*. Thus, the reaction between E·G and S* is first order in E·G (Figure 2), and the second-order rate constant for this reaction is simply k_{cat}/K_m. The concentration of ribozyme that can partake in the reaction is reduced by the binding of P to form E·G·P (Scheme III), resulting in a decrease in $k_{obsd} = (k_{cat}/K_m)$[E·G], the observed rate constant for the disappearance of S*. Thus, the value of K_i = 0.6 μM obtained in Figure 4 is expected to be equivalent to K_d(E·P). This method for determining values of K_i from experiments under single-turnover conditions, such that the reaction is first order in free ribozyme, is preferable to the standard method used in steady-state kinetic determinations of K_i. The single-turnover method eliminates the requirement to extrapolate the results from multiple experiments to zero substrate concentration and allows determination of the observed rate constant for data over several half-lives without encumbrance from product inhibition.

This value of K_d(E·P) = 0.6 ± 0.2 μM and k_{off}^{P} = 6 ± 2 min^{-1} give $k_{on}^{P} = k_{off}^{P}/K_d$(E·P) = 1 × 10^7 M^{-1} min^{-1} [range (0.5–2) × 10^7 M^{-1} min^{-1}]. This value of k_{on}^{P} is similar to the value of k_{on}^{S} = (2 ± 1) × 10^7 M^{-1} min^{-1}, as expected since the substrate and product both form a base-paired duplex with the 5′ exon binding site.

Michaelis and Dissociation Constants for Guanosine. The dependence on the concentration of G of the rate of the endonuclease reaction of the mismatched substrate with ribozyme in excess is shown in Figure 5. These data give K_m^{G} = 110

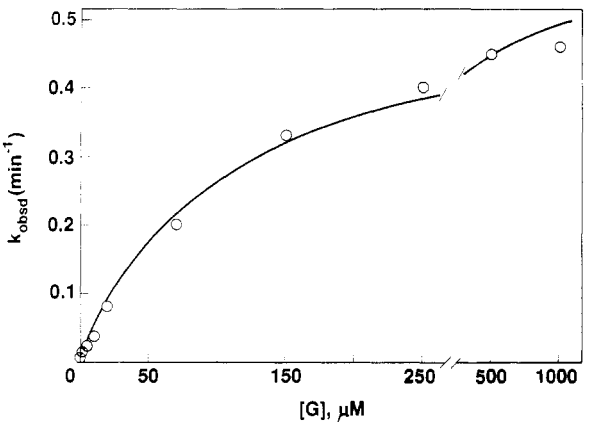


FIGURE 5: Dependence of k_{obsd} on the concentration of guanosine. The reaction of ~1 nM p*G_2CCCGCUA$_5$ was catalyzed by 50 nM ribozyme at 50 °C in 50 mM MES, pH 7, and 10 mM $MgCl_2$. The solid line is the nonlinear least-squares fit to the data that gives K_m^G = 110 μM and k_{obsd} = 0.55 min^{-1} at saturating guanosine. The line does not pass through the y intercept at k_{obsd} = 0 because of the hydrolysis reaction (see text and Herschlag and Cech, preceding paper).

± 30 μM under these conditions, in which essentially all of the S* is free in solution (i.e., the concentration of E is subsaturating; Figure 2).

The value of K_m^G is not equal to the dissociation constant for G, K_d(E·G), but rather reflects in part a change in rate-limiting step from chemistry at low [G] (k_c, Scheme I) to a combination of chemistry and binding of S at saturating G. At sufficiently low G, an E·S binary complex will be more likely to dissociate than to bind G, i.e., $k_{off}^S > k_{on}^G$[G]. Thus, S can dissociate faster than G can bind and react, and the binding of S is not rate-limiting. (The free energy of the barrier for binding is, of course, the same as that for dissociation, so that faster dissociation than chemistry means that chemistry, not binding, is rate-limiting.) At high G concentration (800 μM), binding of S and chemistry are each partially rate-limiting as evidenced by the pulse–chase experiments described above (Figure 3). If binding of S were faster and did not impose an upper limit on the rate, then the rate would continue to increase at higher concentrations of G so that saturation would not occur until higher concentrations of G. However, since the rate of binding of S does impose such an upper limit, saturation occurs at lower concentration of G and the observed K_m^G is less than K_d(E·G).

The quantitative relationship of K_m^G to the actual dissociation constant is obtained as follows. The Michaelis–Menten equation for these conditions of varying G and E in excess over S, with [E] ≪ K_d(E·S), is given in eq 6. Note that the second-order rate constant for the reaction of E·G and S at saturating G, k_2^G, is simply k_{cat}/K_m for S. At low concentrations of G the reaction will be third order overall, and first order in E, S, and G, as given in eq 7. Eq 7 was derived for independent binding of G and S (see above and Herschlag and Cech, preceding paper). Combining eqs 3, 4, 6, and 7 gives eq 8, which relates the values of K_m^G and K_d(E·G). Note that

$$\text{vel(at subsaturating S)}/[S] = k_2^G[G][E]/(K_m^G + [G]) \quad (6)$$

$$\text{vel(at low concentrations of G, S, and E)} = k_3[G][E][S] \qquad k_3 = k_c/K_d(E{\cdot}G)K_d(E{\cdot}S) \quad (7)$$

$$K_m^G = K_d(E{\cdot}G)k_{off}^S/(k_c + k_{off}^S) \quad (8)$$

with $k_{off}^S \gg k_c$ the central step is fully rate-limiting and K_m^G = K_d(E·G). In order to solve eq 8, the value of k_c is needed. The expression of eq 2 is also dependent on k_c so that this expression can be used to algebraically eliminate k_c and solve eq 8 for K_d(E·G). Substitution of the expression for k_c for eq 2 into eq 8 and simplification using eq 3 give eq 9 for K_d(E·G).

$$K_d(E{\cdot}G) = \frac{[G]}{\{[G]/K_m^G\}\{1 - (\text{fracP*})_{max}\} - (\text{fracP*})_{max}} \quad (9)$$

Solving eq 9 gives K_d(E·G) = 0.5 mM with use of K_m^G = 110 μM, [G] = 800 μM, and (fracP*)$_{max}$ = 0.7 (from Figures 3 and 5). Because this calculation involves data from two different experiments, there is considerable uncertainty in this value of K_d(E·G). We estimate an uncertainty of K_d(E·G) ≅ 0.3–1.1 mM from calculations with the extreme values of K_m^G of 80 and 140 μM. Because of the evidence for independent binding of substrate and G, K_d(E·G) ≅ 0.5 mM is used in Scheme I of the preceding paper (Herschlag & Cech, 1990b) for the matched substrate as well. These calculations assume that reaction of the ternary complex, not binding of G, limits the rate for reaction of E·S (i.e., G dissociates faster than k_c). Rate-limiting binding of G would require binding to occur several orders of magnitude slower than diffusion (see Herschlag and Cech, preceding paper). The validity of this assumption is also supported by the similarity of K_m^G = 1 mM for the slow DNA endonuclease reaction (Herschlag & Cech, 1990a) to the calculated K_d(E·G) ≅ 0.5 mM (see Discussion).

Estimation of the Rate Constant for Reaction of the Ternary Complex. The value of k_c = 200 min^{-1} for the chemical step of the ternary complex (Scheme I) is obtained from the value of K_d(E·G) = 0.5 mM, k_c^{obsd} = 120 min^{-1}, and [G] = 800 μM with use of eq 2. Again, there is a large range of uncertainty with k_c = 70–500 min^{-1} calculated from the extremes of K_d(E·G) = 0.3 and 1.1 mM and the extreme of k_c^{obsd} = 50 and 210 min^{-1}. This calculation of k_c assumes that the binding of G is not rate-limiting, as described above. The chemical step includes chemistry and any conformational changes of the ternary complex that limit the rate. As there is no evidence for rate-limiting conformational steps, we have adopted the simplest model in which k_c represents the rate of chemistry (see Herschlag and Cech, preceding paper).

Dissociation Constant for G_2CUCUCU. The 5′ exon from *Tetrahymena* pre-rRNA has the 3′-terminal sequence CUCUCU, so that U(−5) forms a wobble pair with a G of the 5′ exon binding site (Figure 1). The dissociation constant for pppG$_2$CUCUCU was obtained by inhibition of the endonuclease reaction using the same procedure outlined above for the mismatched product, pppG$_2$CCCGCU (Figure 4). Inhibition by 20–500 nM pppG$_2$CUCUCU of the reaction of 10 nM E, ~1 nM S*, and 800 μM G gave K_i = K_d = 25 (±~10) nM (data not shown). The same value of K_d was obtained in an experiment with 20 nM ribozyme.

The 5′-terminal G residues were cleaved from pppG$_2$CUCUCU and pppG$_2$CCCUCU by RNase T1 to form CUCUCU and CCCUCU, respectively. Removal of the G residues, which do not base pair with the 5′ exon binding site (Figure 1), resulted in only a 3–5-fold increase in the values of K_d obtained in inhibition experiments (data not shown). This type of modest destabilization is expected from the loss in stacking interactions from the removal of the terminal mismatch (Freier et al., 1986).

Discrimination between Matched and Mismatched Substrates. The open symbols in Figure 6 show product formation in the reaction of the matched substrate, G$_2$CCC*U*CUA$_5$, and the mismatched substrate, G$_2$CCC*G*CUA$_5$, in the presence of 800 μM G during a single turnover with 10 nM ribozyme. The mismatched substrate reacted only ~8-fold slower than the matched substrate, consistent with the 5-fold difference in the

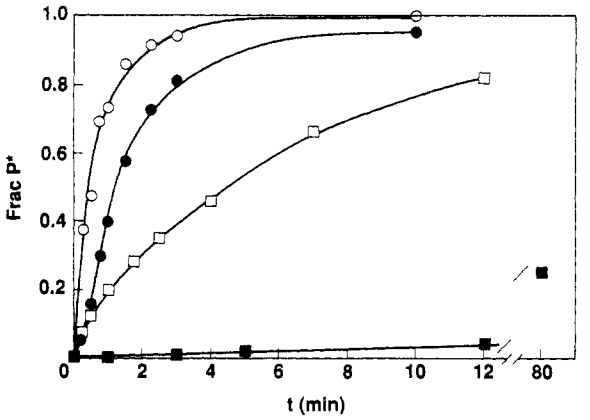


FIGURE 6: Reaction of matched substrate (p*G_2CCC*U*CUA_5; circles) and mismatched substrate (p*G_2CCC*G*CUA_5; squares) with 800 μM G (open symbols) or 1 μM G (closed symbols). The fraction of S* converted to P* is plotted as a function of time for reactions of ~1 nM S* with 10 nM ribozyme, 50 mM MES, pH 7, and 10 mM $MgCl_2$ at 50 °C.

values of k_{cat}/K_m listed in Table I. (The data of Table I represent average values from several experiments.) However, lowering the concentration of G to 1 μM (Figure 6, closed symbols) greatly slowed the reaction of the mismatched substrate but had little effect on the reaction of the matched substrate. Thus, the ratio of the observed rates of ~200 is much larger than obtained at the higher concentration of G. These reactions with 10 nM ribozyme and ~1 nM S* are under subsaturating or "k_{cat}/K_m" conditions, meaning that the reactions are first order in both E and S (see Figure 2 and Herschlag and Cech, preceding paper). The ratio of k_{cat}/K_m values determines the discrimination between two substrates (see Discussion), so that the data of Figure 6 show that specificity is enhanced at low concentrations of G.

Discussion

The *Tetrahymena* ribozyme catalyzes a site-specific endonuclease reaction with matched and mismatched substrates (Figure 1). We have compared the reactions of these substrates as an initial step toward understanding the catalytic power of this ribozyme. More specifically, the comparison reveals the contribution of a base pair to binding and to catalysis. These data have implications for the accuracy of 5′ splice site selection. From a practical perspective, these data enable understanding and improvement of the specificity of these site-specific RNA endonucleases. The "hammerhead" and "hairpin" ribozymes also recognize substrates by base pairing (Forster & Symons, 1987; Uhlenbeck, 1987; Ruffner et al., 1989; Hampel et al., 1990), so that similar analyses should allow the specificity of these ribozymes to be evaluated and understood.

Our characterization of the reaction with the mismatched substrate is summarized in Scheme I and Table I. This reaction, with saturating G, proceeds with $k_{cat}/K_m = 2 \times 10^7$ M^{-1} min^{-1} and $k_{cat} = 6$ min^{-1}. The similar rate constants for dissociation of substrate and reaction of the E·S·G ternary complex determined in pulse–chase experiments (Figure 3) show that binding of substrate, k_{on}^S, and the chemical step, k_c, are each partially rate-limiting for k_{cat}/K_m. The large value of $k_c \cong 200$ min^{-1} relative to $k_{cat} = 6$ min^{-1} suggests that dissociation of the product corresponding to the 5′ portion of the substrate is rate-limiting for k_{cat}.

The value of k_{cat}/K_m for the mismatched substrate is similar to that for the matched substrate (Table I), because at low substrate concentrations binding to form the ribozyme/substrate helix partially limits the rate for the mismatched substrate and completely limits the rate for the matched substrate (Herschlag and Cech, preceding paper). The value of k_{cat} is ~10^2-fold larger for the mismatched substrate, because with saturating substrate the dissociation of the product is rate-limiting with both substrates and binding of the mismatched product is much weaker (K_d = 1 and 600 nM for the matched and mismatched product, respectively).

Binding of S and binding of G are random and independent in Scheme I. Product release can occur with or without G bound to E·P, since GA_5 presumably dissociates fast and matched product and G bind essentially independently (see Results and Herschlag and Cech, preceding paper).

"Uniform Binding" of the Substrate. The rate constant k_c for the chemical step is within ~2-fold for the matched and mismatched substrate in both the G-dependent and independent reactions, despite the ~10^3-fold difference in affinity for these substrates ($k_c \cong 350$ and 200 min^{-1}; $k_c(-G) = 0.7$ and 0.4 min^{-1}; and K_d = 2 and 2500 nM for the matched and mismatched substrate, respectively). Thus, the stabilization from the base pairing interaction at position −3 (Figure 1) is the same in the ground state and transition state. This can be described as "uniform binding" in the ground and transition states (Albery & Knowles, 1976). The uniform binding shows that the extra energy from this base pair cannot be used to preferentially stabilize the transition state. In contrast, comparison of the ribozyme reactions with RNA and DNA substrates shows that one or more 2′-hydroxyls provide preferential transition-state stabilization (Herschlag & Cech, 1990a). Such preferential stabilization can result from the changes in going from the tetrahedral ground state to the trigonal bipyramidal transition state [e.g., Leatherbarrow et al. (1985)] from coordination of a catalytic metal ion, or from general-acid–base catalysis. Both uniform binding and preferential transition-state stabilization have been observed with protein enzymes. For example, the adenosine 3′-phosphate 5′-diphosphate moiety of coenzyme A exhibits uniform binding in the reaction of CoA transferase, whereas the pantetheine moiety provides preferential transition-state stabilization (Fierke & Jencks, 1986; Jencks, 1987).

Several "hammerhead" ribozymes that have different substrate recognition sequences react with similar values of k_{cat} $\cong$ 1 min^{-1} (Fedor & Uhlenbeck, 1990). The similarity of k_{cat} values is consistent with a role for the hammerhead/substrate base pairs in binding, but not in preferential stabilization of the transition state for the rate-limiting step. This can be described as "uniform binding" by the hammerhead ribozymes, as for the *Tetrahymena* ribozyme, although it is not known whether k_{cat} in the hammerhead reactions represents the rate of chemistry or of a different step.

Effect of Alterations in the Base Pairing at the 5′ Exon Binding Site and a Model for Binding. Table II summarizes the observed dissociation constants for complexes between oligonucleotides and the ribozyme (50 °C, 10 mM $MgCl_2$). These values are compared to those predicted from nearest-neighbor rules for helices between oligonucleotides (Freier et al., 1986). Each of the oligonucleotides binds ~10^4-fold stronger than calculated for a simple helix. [The difference in stability varies with temperature (Pyle et al., 1990).] This strongly suggests that tertiary interactions stabilize the binding of substrate. Furthermore, the similar values of $\Delta\Delta G_{obsd}$ and $\Delta\Delta G_{predicted}$ in Table II show that creation of a single mismatch at position −3 or a wobble pair at position −5 (Figure 1) destabilizes binding by about the amount expected on the basis solely of the stability of short helices; the 0.9 kcal/mol difference in $\Delta\Delta G_{obsd}$ and $\Delta\Delta G_{predicted}$ for G_2CUCUCU corre-

Table II: Stability of Complexes of Oligonucleotides with the Ribozyme Compared to the Predicted Stability of the Corresponding Simple RNA Helices

oligonucleotide product	K_d^{obsd} [a] (μM)	$K_d^{predicted}$ [b,c] (μM)	$\Delta G°_{obsd}$ [c] (kcal/mol)	$\Delta G°_{predicted}$ [b] (kcal/mol)	$\Delta\Delta G°_{obsd}$ [d] (kcal/mol)	$\Delta\Delta G°_{predicted}$ [e] (kcal/mol)
(G_2)CCCUCU	0.0009	10	−13.4	−7.4	(0)	(0)
(G_2)CUCUCU	0.025	1200	−11.2	−4.3	2.2	3.1
(G_2)CCCGCU	0.6	6800	−9.2	−3.2	4.2	4.2

[a] Dissociation constants obtained at 50 °C in 50 mM MES, pH 7, and 10 mM $MgCl_2$ as described under Results of this and the preceding paper. [b] Predicted stabilities at 50 °C are for formation of helices with 5′GGAGGG(A), the 5′ exon binding site, calculated with use of rules for RNA helix stability based on the free energy parameters of Freier et al. (1986) and D. Turner (personal communication). The contribution to the free energy from helix initiation at 50 °C of +3.5 kcal/mol has been included in the predicted values. [c] Free energy values and dissociation constants were interconverted with use of the equation $\Delta G° = -RT \ln (1/K_d)$. [d] The difference in the observed binding free energy relative to that observed for (G_2)CCCUCU. [e] The difference in the predicted binding free energy relative to that predicted for (G_2)CCCUCU.

sponds to a 4-fold difference in K_d.

These data are consistent with a simple RNA·RNA duplex that holds the substrate in position to allow additional interactions with the ribozyme at the reactive phosphoryl group that are crucial to catalysis. The much weaker binding of the deoxyoligonucleotide, deoxy-(CCCUCU), with K_d = 20 μM is similar to that predicted for a simple *RNA*·RNA duplex (Table II) and is consistent with stabilization of binding of the RNA substrates by tertiary interactions of the ribozyme that involve one or more 2′-hydroxyls (Herschlag & Cech, 1990a).

It is possible that some or all of the extra binding energy occurs because tertiary interactions hold the 5′ exon binding site in place, thereby eliminating the requirement for a loss in entropy from aligning the binding site into a helical conformation. An analogous model proposed to account for the strong binding of mRNA codons to the anticodons of tRNA molecules may provide a precedent for this mechanism (Uhlenbeck et al., 1970; Hogenauer, 1970; Eisenger et al., 1971; Labuda et al., 1984); however, Yoon et al. (1976) have suggested that stacking of non-base-paired nucleotides can account for the strong binding in this case. One might expect that removal of the entropic barrier of binding would enhance binding of both RNA and DNA to the ribozyme, whereas only the binding of RNA appears to be greatly enhanced. However, helix geometry that specifically favors RNA binding cannot be ruled out (Herschlag & Cech, 1990a).

Sugimoto et al. (1988, 1989) have suggested that the extra stabilization involves interactions with the oligonucleotide at positions −1 and −2 from the reaction site. It should be noted that the binding site for the oligonucleotides in the circle-opening reaction studied by Sugimoto et al. is different from that in splicing and the endonuclease reaction; the site in circle opening is displaced 3–4 bases in the 3′ direction (Been & Cech, 1986, 1987). Thus, the binding interactions in these reactions might not be governed by the same rules.

The Value of K_m^G Is Not Equal to the Value of K_d(E·G): Implications. There is a change in the rate-limiting step in going from subsaturating to saturating G in the reaction of subsaturating mismatched substrate. This change in rate-limiting step causes K_m^G = 110 μM to be less than the dissociation constant of K_d(E·G) ≅ 0.5 mM, as described under Results. This measurement assumes that the binding of G is not rate-limiting, which is reasonable as binding would need to be several orders of magnitude slower than diffusion-controlled in order to be rate-limiting. In addition, the value of K_m^G = 1 mM for the DNA endonuclease reaction catalyzed by this ribozyme with subsaturating DNA substrate (Herschlag & Cech, 1990a) is the same, within error, as the value of K_d(E·G) ≅ 0.5 mM determined indirectly in the present work. The K_m^G for the DNA reaction presumably equals K_d(E·G) as the reaction is slow and analogous to the RNA endonuclease reactions studied in this and the preceding paper (Herschlag & Cech, 1990b). Determination of the same value of K_d(E·G), within error, from the two different reactions provides support for the conclusion that K_d(E·G) ≅ 0.5–1 mM is a true dissociation constant. This agreement provides an indication that Scheme I is an adequate description of the kinetics for cleavage of the mismatched substrate. This value of K_d(E·G) ≅ 0.5–1 mM is the first measurement of a true dissociation constant for G bound to the IVS of which we are aware, though it is subject to considerable experimental uncertainty (see Results). Measurement of K_d(E·G) represents a necessary step toward a detailed understanding of the interactions at the guanosine binding site that mediate binding and catalysis.

As with subsaturating oligonucleotide substrate, the value of K_m^G = 15 μM with saturating oligonucleotide substrate (Table I) is also not equal to K_d(E·G) of ~0.5 mM. This low K_m relative to K_d is also due to a change in rate-limiting step, in this case, from the chemical conversion with low concentrations of G to product release with high concentrations of G. In general, saturation behavior can result from complex formation or from a change from a bi- (or tri-) molecular rate-limiting step to a uni- (or bi-) molecular rate-limiting step, even without significant complex formation (Jencks, 1969).

It has been assumed that the value of K_m^G could be equal to the dissociation constant for G in several reactions of the *Tetrahymena* IVS, because the small observed rate constant at saturating G should allow G to bind and dissociate many times before a slow chemical step in a simple Michaelis–Menten reaction scheme (Bass & Cech, 1986; Tanner & Cech, 1987; Michel et al., 1989). However, even though G presumably dissociates fast relative to the rate of the chemical step in the endonuclease reaction, the value of K_m^G is not equal to K_d(E·G). Other reactions that are slow could still have a change in rate-limiting step that results in $K_m^G < K_d$(E·G). There could be a G-independent conformational change that is rate-limiting at saturating but not subsaturating G. Indeed, there is evidence for a slow conformational change in the opening of a circular form of the IVS that has been purified by denaturing PAGE (S. Walstrum and O. C. Uhlenbeck, personal communication). In the self-splicing reaction, the relation of the Michaelis and dissociation constants for G remains to be determined.

Specificity of the Tetrahymena Ribozyme. The discrimination between two competing substrates is determined by the ratio of the values of k_{cat}/K_m and the respective concentrations of the substrates according to eq 10 (Fersht, 1985). The ratio

$$\text{relative rates of reaction} = (k_{cat}/K_m)^A[A]/(k_{cat}/K_m)^B[B] = (\text{specificity constant})[A]/[B] \quad (10)$$

of the values of k_{cat}/K_m is referred to as the "specificity constant". The similar values of k_{cat}/K_m for the matched and mismatched substrates with saturating G show that there is little specificity under these reaction conditions, despite the

$\sim 10^3$-fold difference in binding affinity: $(k_{cat}/K_m)^{match}/(k_{cat}/K_m)^{mismatch} = (9 \times 10^7\ M^{-1}\ min^{-1})/(2 \times 10^7\ M^{-1}\ min^{-1}) = 4.5$ (Table I). This similarity in the values of k_{cat}/K_m with saturating G is demonstrated by the reaction profiles with matched and mismatched substrate shown in Figure 6 (open symbols). The low specificity occurs because each substrate reacts almost every time it binds; the difference in binding affinity, which is manifest in different values of k_{off}^S, has a very small effect on k_{cat}/K_m and the specificity.

However, the reaction profiles for matched and mismatched substrate with 1 μM G (Figure 6; closed symbols) show that the values of k_{cat}/K_m at low G are very different so that there is good discrimination. The discrimination occurs because there is a change in rate-limiting step for the mismatched substrate at low concentrations of G, as described in the previous section. At the low concentrations of G there is less ternary complex and slower reaction so that the mismatched substrate dissociates faster than it reacts; in contrast, the matched substrate still reacts nearly at its rate of binding. Rapid dissociation of the mismatched substrate relative to the rate of the chemical conversion, i.e., near-equilibrium binding of the substrate prior to reaction, allows discrimination between the matched and mismatched substrates based on the different affinity of the substrates.

This can be demonstrated with a numerical example, using the conditions of Figure 6. With 1 μM G the rate of the forward reaction of E·S for the matched substrate is, according to eq 11, $k_c^{app} = 0.7\ min^{-1} + (7 \times 10^5\ M^{-1}\ min^{-1})(1\ \mu M) = 1.4\ min^{-1}$. This is $\sim$10-fold larger than the rate constant for dissociation of S from E·S of $k_{off}^S = 0.2\ min^{-1}$ (Herschlag and Cech, preceding paper) so that the matched substrate will still react almost every time it binds. The observed value of $(k_{cat}/K_m)^{matched}_{obsd} = 8 \times 10^7\ M^{-1}\ min^{-1}$ with [G] = 1 μM is calculated from k_c^{app}, k_{off}^S, and $k_{on}^S = 9 \times 10^7\ M^{-1}\ min^{-1}$ (Herschlag and Cech, preceding paper) with use of eq 12. The

$$k_c^{app} = k_c(-G) + (k_{cat}/K_m)^G[G] \qquad [G] \ll K_d(E{\cdot}G) \tag{11}$$

$$(k_{cat}/K_m)^S_{obsd} = k_{on}^S k_c^{app}/(k_c^{app} + k_{off}^S) \tag{12}$$

analogous calculations for the mismatched substrate give the apparent value of $(k_{cat}/K_m)^{mismatched}_{obsd} = 4 \times 10^5\ M^{-1}\ min^{-1}$ with 1 μM G, according to eq 12, with $k_c^{app} = 0.4\ min^{-1} + (5 \times 10^5\ M^{-1}\ min^{-1})(1\ \mu M) = 0.9\ min^{-1}$ (eq 11), $k_{on}^S = 2 \times 10^7\ M^{-1}\ min^{-1}$, and $k_{off}^S = 50\ min^{-1}$ (rate constants from Scheme I and Table I). Thus, the "specificity constant" (eq 10) with 1 μM G is $(8 \times 10^7\ M^{-1}\ min^{-1})/(4 \times 10^5\ M^{-1}\ min^{-1}) = 200$. This value, which is derived from the kinetic scheme for the matched and mismatched substrates, agrees with the observed specificity of 210-fold from the data of Figure 6 (first-order plots not shown).

An increase in the specificity of the *Tetrahymena* ribozyme at high concentrations of G upon addition of urea has previously been observed (Zaug et al., 1986). The kinetic mechanisms derived in this and the preceding paper for a mismatched and matched substrate (Herschlag & Cech, 1990b) suggest the origin of this enhanced specificity. The addition of urea could have two effects that would enhance specificity: (1) Urea weakens base pairing interactions (Lerman et al., 1984). This would increase the rate constant for dissociation of the mismatched and the matched substrate. (2) The addition of urea could disrupt the structure of the ribozyme and decrease the rate of the chemical step. Increasing the rate of dissociation and decreasing the rate of the chemical step would each allow binding of the substrate to be closer to equilibrium prior to reaction. Thus, in the presence of urea the differential stability of the matched and mismatched substrate/ribozyme helices can be reflected in the values of k_{cat}/K_m and in the specificity constant (eq 10).

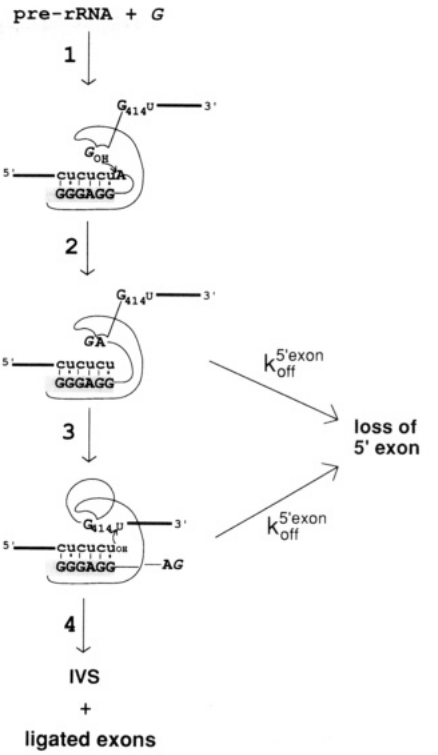


FIGURE 7: Tight binding of the 5′ exon ensures efficient self-splicing. A scheme for self-splicing is shown (Inoue et al., 1986), including the nonproductive pathway of 5′ exon loss. (1) Binding of the G cofactor. (2) The first transesterification step, attack by G at the 5′splice site. This leaves the 5′ exon bound at the 5′ exon binding site, but no longer covalently attached. (3) The G cofactor, which is now covalently attached to the 5′ end of the IVS, exits the guanosine binding site and is replaced by G_{414}, which directly precedes the 3′ exon (Michel et al., 1989). (4) The second transesterification step, in which the 5′ exon attacks after G_{414} to give ligated exons and free IVS. [This step is analogous to the reverse of the first transesterification step (2), but with G_{414} replacing the exogenous G (Inoue et al., 1986).] Because the 5′ exon is not covalently attached after steps 2 and 3, the 5′ exon could dissociate and be lost to solution ($k_{off}^{5'exon}$), thereby preventing exon ligation. The intervening sequence is represented by the large upper case letters and the thin line; the exon sequences are represented by the small upper case letters and the thick lines; the 5′ exon binding site, a portion of the "internal guide sequence", is shaded; and the exogenous guanosine cofactor is represented by the italic G.

Implications for the Efficiency of Splicing. The very strong binding of the oligonucleotide containing the 3′-terminal sequence of the 5′ exon, (G_2)CUCUCU, and the weaker binding of G (K_d = 0.025 and 500 μM, respectively) are consistent with the biological role of the IVS, which is the catalysis of a single self-excision event rather than catalysis of multiple turnover in an endonuclease reaction. In the first transesterification reaction of self-splicing the bond to the 5′ exon is broken (Figure 7, step 2). At this intermediate stage, loss of the 5′ exon ($k_{off}^{5'exon}$) is prevented by the strong binding interactions, thereby ensuring efficient splicing. The half-life for the 5′ exon·IVS complex is estimated to be 30 s [on the basis of the K_d for G_2CUCUCU from Table II and the assumption that $k_{on}(G_2CUCUCU)$ is similar to the measured values for G_2CCCUCU and G_2CCCGCU (Scheme I and Herschlag and Cech, preceding paper)]. The weaker binding at the G site can also aid efficient splicing by ensuring that the G that has added to the 5′ end of the IVS leaves the active site before the 5′ exon. According to the model of Figure 7,

G_{414} at the 3′ splice site can then enter the active site (step 3). Furthermore, the fast rate of the chemical step in the endonuclease reaction of $k_c \cong 200$–350 min^{-1} ($t_{1/2} \cong 0.1$ s) is consistent with the IVS catalyzing exon ligation considerably faster than dissociation occurs ($t_{1/2} \cong 30$ s; Figure 7, $k_{off}^{5'exon}$ and step 4).

The slow dissociation of the 5′ exon analogue G_2CUCUCU, with $t_{1/2} \cong 30$ s, arises from two factors. (1) The extra 6 kcal/mol binding energy beyond that predicted for a simple RNA·RNA duplex (Table II) increases binding and slows dissociation by 10^4-fold. Without the thermodynamic barrier to dissociation provided by this extra binding energy, dissociation of G_2CUCUCU would occur 10^4-fold faster, with $t_{1/2} \cong 0.001$ s. (2) As described in the preceding paper (Herschlag and Cech, 1990b), oligonucleotides bind to the ribozyme $\sim 10^2$-fold slower than the diffusion-controlled limit, consistent with rate-limiting helix nucleation in binding. This kinetic barrier to binding also provides a kinetic barrier to dissociation of 10^2-fold because the lowest energy pathway for binding, via nucleation, is also the lowest energy pathway for dissociation (i.e., microscopic reversibility holds).

ACKNOWLEDGMENTS

We thank Rob Kuchta for helpful discussions; John Latham, Jim McSwiggen, and Art Zaug for gifts of materials; and Doug Turner for comments on the manuscript.

REFERENCES

Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5631–5640.
Bass, B. L., & Cech, T. R. (1986) *Biochemistry 25*, 4473–4477.
Been, M. D., & Cech, T. R. (1986) *Cell 47*, 207–216.
Been, M. D., & Cech, T. R. (1987) *Cell 50*, 951–961.
Been, M. D., & Cech, T. R. (1988) *Science 239*, 1412–1416.
Cech, T. R., & Bass, B. L. (1986) *Annu. Rev. Biochem. 55*, 599–629.
Davies, R. W., Waring, R. B., Ray, J. A., Brown, T. A., & Scazzocchio, C. (1982) *Nature 300*, 719–724.
Eigen, M., & Hammes, G. G. (1963) *Adv. Enzymol. 25*, 1–38.
Eisenger, J., Feuer, B., & Yamane, T. (1971) *Nature, New Biol. 231*, 126–128.
Fedor, M., & Uhlenbeck, O. C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1668–1672.
Fersht, A. (1985) *Enzyme Structure and Mechanism*, 2nd ed., pp 111–112, W. H. Freeman, New York.
Fierke, C. A., & Jencks, W. P. (1986) *J. Biol. Chem. 261*, 7603–7606.
Forster, A. C., & Symons, R. H. (1987) *Cell 50*, 9–16.
Freier, S. M., Kierzek, R., Sugimoto, N., Caruthers, M. H., Neilson, T., & Turner, D. H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 9373–9377.
Good, N. E., Winget, D., Winter, W., Connolly, T. N., Izawa, S., & Singh, R. M. M. (1966) *Biochemistry 2*, 467–477.
Hampel, A., Tritz, R., Hicks, M., & Cruz, P. (1990) *Nucleic Acids Res. 18*, 299–304.
Herschlag, D., & Cech, T. R. (1990a) *Nature 344*, 405–409.
Herschlag, D., & Cech, T. R. (1990b) *Biochemistry* (preceding paper in this issue).
Hogenauer, G. (1970) *Eur. J. Biochem. 12*, 527–532.
Inoue, T., Sullivan, F. X., & Cech, T. R. (1986) *J. Mol. Biol. 189*, 143–165.
Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, pp 571–576, McGraw Hill, New York.
Jencks, W. P. (1987) *Cold Spring Harbor Symp. Quant. Biol. 52*, 65–73.
Kruger, K., Grabowski, P. J., Zaug, A. J., Sands, J., Gottschling, D. E., & Cech, T. R. (1982) *Cell 31*, 147–157.
Labuda, D., Striker, G., & Porschke, D. (1984) *J. Mol. Biol. 174*, 587–604.
Leatherbarrow, R. J., Fersht, A. R., & Winter, G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7840–7844.
Lerman, L. S., Fischer, S. G., Hurley, I., Silverstein, K., & Lumelsky, N. (1984) *Annu. Rev. Biophys. Bioeng. 13*, 399–423.
Michel, F., Jacquier, A., & Dujon, B. (1982) *Biochimie 64*, 867–881.
Michel, F., Hanna, M., Green, R., Bartel, D. P., & Szostak, J. W. (1989) *Nature 342*, 391–395.
Murphy, F. L., & Cech, T. R. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9218–9222.
Pyle, A. M., McSwiggen, J., & Cech, T. R. (1990) *Proc. Natl. Acad. Sci. U.S.A.* (in press).
Ruffner, D. E., Dahm, S. C., & Uhlenbeck, O. C. (1989) *Gene 82*, 31–41.
Sugimoto, N., Kierzek, R., & Turner, D. H. (1988) *Biochemistry 27*, 6384–6392.
Sugimoto, N., Sasaki, M., Kierzek, R., & Turner, D. H. (1989) *Chem. Lett.*, 2223–2226.
Tanner, N. K., & Cech, T. R. (1987) *Biochemistry 26*, 3330–3340.
Uhlenbeck, O. C. (1987) *Nature 328*, 596–600.
Uhlenbeck, O. C., Baller, J., & Doty, P. (1970) *Nature 225*, 508–510.
Waring, R. B., Towner, P., Minter, S. J., & Davies, R. W. (1986) *Nature 321*, 133–139.
Yoon, K., Turner, D. H., Tinoco, I., Jr., von der Haar, F., & Cramer, F. (1976) *Nucleic Acids Res. 3*, 2233–2241.
Zaug, A. J., & Cech, T. R. (1986) *Science 231*, 470–475.
Zaug, A. J., Been, M. D., & Cech, T. R. (1986) *Nature 324*, 429–433.
Zaug, A. J., Grosshans, C. A., & Cech, T. R. (1988) *Biochemistry 27*, 8924–8931.