Catalysis of RNA Cleavage by the *Tetrahymena thermophila* Ribozyme. 1. Kinetic Description of the Reaction of an RNA Substrate Complementary to the Active Site†

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ABSTRACT: A ribozyme derived from the intervening sequence (IVS) of the *Tetrahymena* preribosomal RNA catalyzes a site-specific endonuclease reaction: $G_2CCCUCUA_5 + G \rightleftharpoons G_2CCCUCU + GA_5$ (G = guanosine). This reaction is analogous to the first step in self-splicing of the pre-rRNA, with the product $G_2CCCUCU$ analogous to the 5′-exon. The following mechanistic conclusions have been derived from pre-steady-state and steady-state kinetic measurements at 50 °C and neutral pH in the presence of 10 mM Mg^{2+}. The value of $k_{cat}/K_m = 9 \times 10^7$ M^{-1} min^{-1} for the oligonucleotide substrate with saturating G represents rate-limiting binding. This rate constant for binding is of the order expected for formation of a RNA·RNA duplex between oligonucleotides. (Phylogenetic and mutational analyses have shown that this substrate is recognized by base pairing to a complementary sequence within the IVS.) The value of $k_{cat} = 0.1$ min^{-1} represents rate-limiting dissociation of the 5′-exon analogue, $G_2CCCUCU$. The product GA_5 dissociates first from the ribozyme because of this slow off-rate for $G_2CCCUCU$. The similar binding of the product, $G_2CCCUCU$, and the substrate, $G_2CCCUCUA_5$, to the 5′-exon binding site of the ribozyme, with K_d = 1–2 nM, shows that the pA_5 portion of the substrate makes no net contribution to binding. Both the substrate and product bind ~10^4-fold (6 kcal/mol) stronger than expected from base pairing with the 5′-exon binding site. Thus, tertiary interactions are involved in binding. Binding of $G_2CCCUCU$ and binding of G are independent. These and other data suggest that binding of the oligonucleotide substrate, $G_2CCCUCUA_5$, and binding of G are essentially random and independent. The rate constant for reaction of the ternary complex is calculated to be $k_c \cong 350$ min^{-1}, a rate constant that is not reflected in the steady-state rate parameters with saturating G. The simplest interpretation is adopted, in which k_c represents the rate of the chemical step. A site-specific endonuclease reaction catalyzed by the *Tetrahymena* ribozyme in the absence of G was observed; the rate of the chemical step with solvent replacing guanosine, $k_c(-G) = 0.7$ min^{-1}, is ~500-fold slower than that with saturating guanosine. The value of $k_{cat}/K_m = 6 \times 10^7$ M^{-1} min^{-1} for this hydrolysis reaction is only slightly smaller than that with saturating guanosine, because the binding of the oligonucleotide substrate is predominantly rate-limiting in both cases. This ribozyme, which approaches the limiting values of k_{cat}/K_m for protein enzymes, can be considered to have achieved "catalytic perfection" [Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5631–5640].

The nuclear pre-rRNA from *Tetrahymena thermophila* can excise its 413-base IVS[1] in the absence of added protein in vitro (Cech et al., 1981; Kruger et al., 1982). In this two-step transesterification reaction, shown in Figure 1A, guanosine (G) first attacks at the 5′-splice site, leaving the 5′-exon with a 3′-hydroxyl group; the second step is analogous to the reverse of the first step, with the 5′-exon attacking the 3′-splice site after a G residue (Cech, 1987). The excised IVS undergoes a series of intramolecular transformations (Cech & Bass, 1986; Inoue et al., 1986). The shortened IVS produced in these reactions can still catalyze reactions when provided with exogenous substrates (Zaug & Cech, 1986). These intra- and intermolecular reactions catalyzed by the IVS are analogous to the splicing reaction.

One shortened form of the IVS, the L-21 *Sca*I RNA, catalyzes reactions such as that shown in eq 1 (Zaug et al., 1988). This reaction is analogous to the first step of splicing, with the oligonucleotide in trans taking the place of the 5′-splice site in the first step of splicing (Figure 1).

$$G_2CCCUCUA_5 + G \xrightleftharpoons{\text{L-21 } Sca\text{I}} G_2CCCUCU_{OH} + GA_5 \quad (1)$$

Data from phylogenetic comparisons and in vitro mutagenesis show that the IVS recognizes the 5′-exon (and the oligonucleotide substrate of eq 1) by base pairing with a 5′-exon binding site (Figure 1B), a portion of the "internal guide sequence" (Davies et al., 1982; Michel et al., 1982; Waring et al., 1986; Been & Cech, 1986; Zaug et al., 1986; Murphy & Cech, 1989). However, binding constants of oligonucleotide substrates and the L-21 *Sca*I ribozyme determined with nondenaturing polyacrylamide gels are several orders of magnitude larger than predicted simply from base pair formation (Pyle et al., 1990). A similar situation has been described for binding of oligonucleotides to a circular form of the same IVS (Sullivan & Cech, 1985; Sugimoto et al., 1988, 1989).

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

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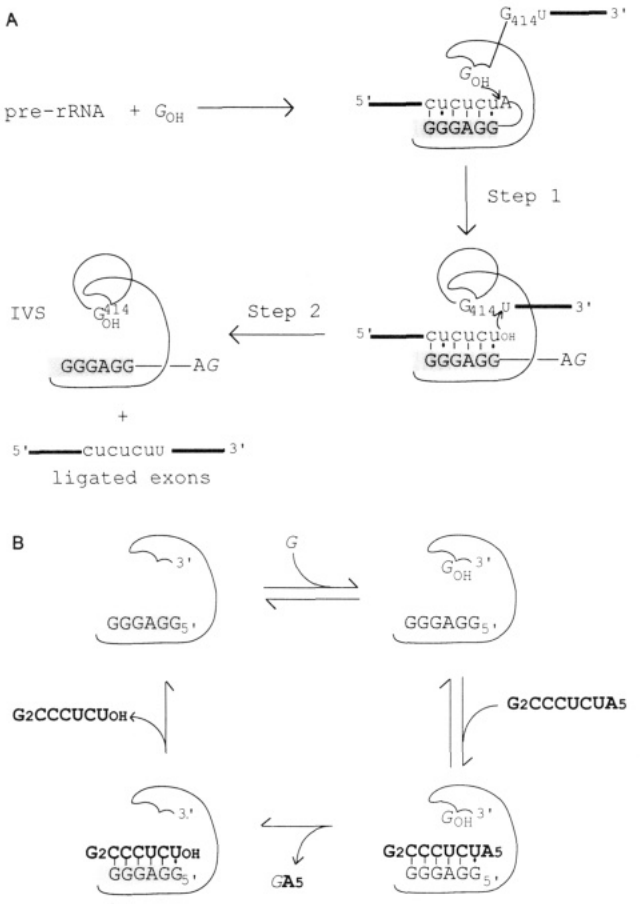


FIGURE 1: (A) Pathway for self-splicing of *Tetrahymena* pre-rRNA via two transesterification steps, as described in the text. Small letters denote the exons; capital letters, IVS; italic G, exogenous guanosine or GTP. (B) Pathway for cleavage of an oligonucleotide substrate by the L-21 *Sca*I ribozyme, a shortened version of the *Tetrahymena* intervening sequence RNA (Zaug et al., 1988). This ribozyme has been engineered so that the sites of intramolecular cleavage and the 3′-terminal G_{414}, which can act as a nucleophile, are absent (Cech, 1987). From Herschlag and Cech (1990a) by permission of Macmillan Journals.

The rate and in some cases the specificity of cleavage of RNA by the L-21 *Sca*I ribozyme have been observed to be affected by the concentration and identity of divalent metal ions, the addition of urea or formamide, the sequence and structure of the RNA substrate, and mutations within the 5′-exon binding site and elsewhere in the molecule (Zaug et al., 1986, 1988; Grosshans & Cech, 1989; Murphy & Cech, 1989; B. Young, D. Herschlag, and T. R. Cech, in preparation; P. Legault, Herschlag, and Cech, unpublished results). Our ability to properly interpret these phenomena has been severely limited by ignorance of the individual step or steps that determine the measured rate constants. The detailed kinetic investigation described herein was initiated to better understand both the binding and catalytic behavior of this ribozyme.

Materials and Methods

Materials. Unlabeled nucleoside triphosphates were purchased from P-L Biochemicals, guanosine was from Sigma, [γ-^{32}P]ATP was from ICN Biomedicals or New England Nuclear, T4 polynucleotide kinase was from United States Biochemicals, calf intestinal phosphatase was from New England Nuclear, RNase T1 was from Sankyo, and *Sca*I restriction endonuclease was from New England Biolabs. Phage T7 RNA polymerase was isolated from *Escherichia coli* strain BL21, containing the plasmid pAR1219 (Davanloo et al., 1984), by A. Zaug.

L-21 ScaI Ribozyme. This ribozyme was prepared essentially as described previously (Zaug et al., 1988). Briefly, plasmid pT7L-21 was cut with *Sca*I restriction endonuclease, ethanol precipitated, and resuspended in H_2O. Transcription by T7 RNA polymerase was followed by purification in a 4% polyacrylamide/8 M urea gel. After visualization by UV shadowing, elution from the gel, and removal of the gel by centrifugation and filtration, the RNA was precipitated in ethanol and chromatographed on a Sephadex G-50 column. Fractions with RNA were pooled, ethanol precipitated, and resuspended in 10 mM Tris·HCl and 1 mM EDTA, at pH 7.5. The concentration of ribozyme was determined spectrophotometrically with use of $\epsilon_{260} = 3.2 \times 10^6$ M^{-1} cm^{-1} (Zaug et al., 1988).

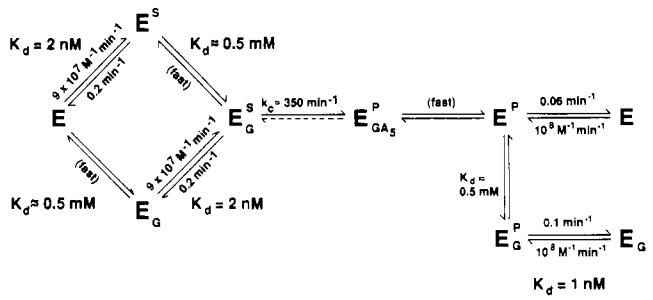
Oligonucleotide Substrate and Product. The RNA substrate was transcribed from a synthetic DNA template by T7 RNA polymerase. The method of Lowary et al. (1986) and Milligan et al. (1987) was employed, as has been described by Zaug et al. (1988). The RNA product, which lacked the terminal five A residues of the substrate, was synthesized in the same way with ATP omitted from the transcription mixture, by J. Latham. Oligonucleotides were purified by electrophoresis on 20% polyacrylamide/7 M urea gels after ethanol precipitation of the transcription mixture. The RNA was visualized by UV shadowing and eluted from the gel; the gel was removed by centrifugation and filtration. The RNA was precipitated in ethanol and resuspended in H_2O or 10 mM Tris and 1 mM EDTA, pH 7.5. Concentrations were determined spectrophotometrically at 260 nm, using an extinction coefficient that is the sum of those for the individual nucleotides (P-L Biochemicals, Circular No. OR-10). Substrate RNA was 5′-end-labeled by treatment with calf alkaline phosphatase followed by polynucleotide kinase and [γ-^{32}P]ATP, essentially as described previously (Zaug et al., 1988). The concentration of labeled substrate was estimated from its specific activity.

Kinetics. All reactions were carried out at 50 °C in 50 mM MES sodium salt, pH 7.0 [determined at 25 °C; pH 6.7 at 50 °C, calculated from Good et al. (1966)], and 10 mM $MgCl_2$. Initial experiments revealed increased rates of reaction when the ribozyme was preincubated with 10 mM $MgCl_2$ in 50 mM MES at 50 °C. The extent of activation was constant from 10 to 60 min. Therefore, reactions were initiated by addition of the oligonucleotide substrate (with or without guanosine) at 50 °C after a 10-min preincubation of the ribozyme, $MgCl_2$, and MES buffer at 50 °C; the presence of guanosine in the preincubation or start mixture had no effect on the activation or subsequent kinetics. Typically, about six aliquots of 1–2 μL were removed from 10-μL reaction mixtures at specified times and quenched with ~2 volumes of 7 M urea and 20 mM EDTA with 0.05% xylene cyanol, 0.1% bromophenol blue, and 1 mM Tris, pH 7.5. Reaction products were separated by electrophoresis on 20% polyacrylamide 7 M urea gels, and the ratio of substrate to product at each time point was quantitated with use of an AMBIS radioanalytic scanner. When reactions were followed for >40 min, the solutions were centrifuged periodically or kept submerged to prevent concentration of the sample by evaporation.

Single-turnover kinetics were performed with excess ribozyme and only radiolabeled substrate. Reactions were first order for ~3 half-lives, and end points were 95–98%; variation in the amount of S* used did not affect the observed rate of reaction, as long as ribozyme excess was maintained.

Rate constants from multiple-turnover experiments were obtained from initial rates, by using the first 15% of the reaction. There was no significant loss in ribozyme activity

Scheme I



during reactions over several hours.

Oligonucleotides used in these reactions that were ^{32}P-labeled had a 5′-phosphate, and those that were not labeled had a 5′-triphosphate.

Pulse–Chase Experiments. "Pulse–chase" experiments were performed as follows. Two microliters of 1.5× ribozyme, $MgCl_2$, and MES was preincubated at 50 °C for 10 min. One microliter of the radiolabeled oligonucleotide substrate was added, such that ribozyme excess was maintained, to start the reaction. The "chase" of 27 μL, which typically contained ~2 μM of the unlabeled substrate or product with 50 mM MES, pH 7.0, and 10 mM $MgCl_2$, was also preincubated at 50 °C. Depending on the experiment, guanosine or GTP was included in or omitted from the chase. The 10-fold dilution and addition of unlabeled substrate or product was designed to prevent reaction of free ribozyme with labeled substrate. In experiments with guanosine or GTP, the amount of labeled product (P*) formed did not vary from 0.25 to 4 min after the chase, confirming that the ribozyme reactions had been effectively quenched by the chase. In preliminary control reactions, the same amount of P* was formed with 500 μM GTP and 2–10 μM unlabeled substrate in the chase, and with 5 μM unlabeled substrate and 500–1000 μM GTP. In the absence of guanosine or GTP the formation of P* is slower (see Results), but the same total amount of P* is formed with 0.8–3.2 μM unlabeled substrate in the chase. This again confirms that the chase is an effective quench.

Estimation of Error Limits. There is good precision in ribozyme-catalyzed reactions performed side by side, with variations in rate constants of <20%, and usually considerably less. However, there is considerably greater variation in experiments performed with different solutions on different days, with variations as large as 2-fold observed. Although some of this error presumably comes from the manipulation of small sample volumes of <10 μL, much of this deviation remains unaccounted for. It should be noted that experiments with ribozyme in excess and saturating, such that increased concentrations of ribozyme did not affect the observed rate of reaction, were more reproducible in independent experiments. The errors reported in the text are, in general, the range of values obtained from independent experiments.

Results

The results of the kinetic investigation described below for the reaction of eq 1 are summarized in Scheme I. For simplicity, the oligonucleotide substrate, $G_2CCCUCUA_5$, is referred to as "substrate" or "S". 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 *Sca*I RNA, is referred to as "E" or "ribozyme". The oligonucleotide product, $G_2CCCUCU$, is referred to as "product" or "P".

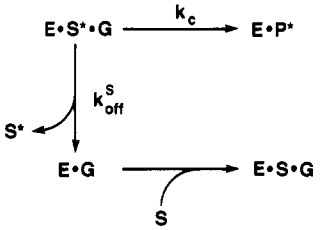
Determination of k_{cat}/K_m for $G_2CCCUCUA_5$. The second-order rate constant for reaction of E·G and S (k_{cat}/K_m) was determined in single-turnover experiments with saturating GTP. The value of k_{obsd} increased linearly with the concentration of E (7–50 nM E, 500 μM GTP). A value of $k_{cat}/K_m = (9 \pm 3) \times 10^7$ M^{-1} min^{-1} was obtained from k_{obsd} divided by [E] (Table I). Replacement of G for GTP had no effect on the value of k_{cat}/K_m. Less precise determination of these parameters by initial rate measurements in multiple-turnover experiments with 0.2 nM E, 0.3–40 nM S, and 500 μM GTP gave values of $k_{cat}/K_m = 5 \times 10^7$ M^{-1} min^{-1} and $k_{cat} = 0.1$ min^{-1} from a reciprocal plot (data not shown), in reasonable agreement with values determined above and below.

Table I: Summary of Steady-State Kinetic Parameters for the Reaction of $G_2CCCUCUA_5$[a]

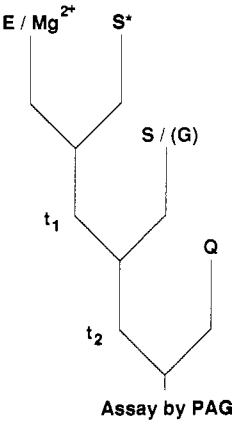
	k_{cat}/K_m (10^7 M^{-1} min^{-1})	k_{cat} (min^{-1})	K_m (μM)	k_c[b] (min^{-1})
with saturating G	9	0.1	0.001	~350
in the absence of G	6	0.05	0.001	0.7

[a] Reactions with 50 mM MES, pH 7, and 10 mM $MgCl_2$, at 50 °C as described under Materials and Methods; experiments carried out as described under Results. [b] The rate constant with saturating G is that for the chemical conversion of the ternary complex E·S·G and in the absence of G is that for the hydrolysis reaction of E·S [$k_c(-G)$ in the text].

Scheme II



Scheme III



The Rate-Limiting Step for k_{cat}/K_m Is Substrate Binding. Pulse–chase experiments were carried out to investigate the partitioning of the ternary E·S·G complex between reaction and dissociation of S (Scheme II; Rose et al., 1974). As outlined in Scheme III, an excess of E was mixed with ^{32}P-labeled substrate (S*) for time t_1 to allow formation of the E·S* complex. G and an excess of unlabeled substance were added to give E·S*·G, which could then partition between the reaction to form product (P*) and irreversible dissociation of S*. The results in Table II show that at least 90% of the E·S* complex proceeds to form product upon addition of GTP or G and unlabeled substrate. The value of 90% is a lower limit because some S* may remain unbound after t_1, and no correction was made for the 2–5% of the S* that is typically unreactive.

These data show that the chemical step (k_c) is much faster than dissociation of S* from the ternary complex, E·S*·G

Table II: Partitioning of E·S*·G (S* = p*G_2CCCUCUA$_5$) between Reaction To Form P* and Dissociation of S* in "Pulse–Chase" Experiments[a]

[E] (nM)	t_1 (min)	fraction of S* trapped as P* after "chase"[b]
100	0.25	0.88[c]
100	0.50	0.89[c]
100	0.33	0.88[c]
200	0.33	0.90[d]

[a] Experimental protocol described in the text and in Scheme III. [b] The amount of S* that formed P* during t_1 (10–30%, depending on the duration of t_1) was determined in control reactions and substracted from the total formation of P* prior to determining the fractions listed in this column. The reaction during t_1 in the absence of G arises from hydrolysis (see text). The amount of P* formed was constant over t_2 (Scheme III) from 0.25 to 4 min. [c] With 500 μM GTP. [d] With 1 mM G.

(k_{off}^S; Scheme II). Since essentially every time substrate binds it will go on to react, the rate-limiting step under k_{cat}/K_m conditions is substrate binding. The formation of labeled product in these experiments also shows that S can bind productively before the binding of G (top path in Scheme I).

The rate of substrate binding was measured directly by using pulse–chase experiments (Scheme III). The time of incubation of E and S*, t_1, was varied, and the amount of E·S* formed in t_1 was assayed by the amount of P* formed following the simultaneous addition of GTP and unlabeled substrate. Figure 2A shows the results of experiments with [E] = 5, 10, 20, and 50 nM. The observed rate constant of P* formation is that for attainment of the equilibrium E + S* ⇌ E·S*, so that $k_{obsd} = k_{on}^S[E] + k_{off}^S$. Figure 2B shows the first-order plots that were used to obtain k_{obsd}. The end points, which were calculated from the concentration of E and K_d(E·S) = 2 nM (see Scheme I and below), give good straight lines in Figure 2B. A value of $k_{on}^S = 10 \times 10^7$ M^{-1} min^{-1} is obtained from the slope of the plot of k_{obsd} against [E] in Figure 2C. The intercept at [E] = 0 is equal to k_{off}^S and is clearly <1 min^{-1}. This value was determined more accurately in separate experiments, as is described below; the point at [E] = 0 in Figure 2C represents the result of that determination.

The value of k_{on}^S determined in this experiment is the same, within error, as the value of $k_{cat}/K_m = 9 \times 10^7$ M^{-1} min^{-1} described above. The value of k_{cat}/K_m was determined in the presence of G, and that for k_{on}^S was determined in its absence, suggesting that the rate of substrate binding is unaffected by the presence of bound G. (Data described below do not support the alternative explanation of ordered binding, with G binding only after S has bound.) The value of $k_{on}^S \cong 10^8$ M^{-1} min^{-1} is significantly less than the rate of diffusional encounter of $\sim 10^{11}$ M^{-1} min^{-1} (Eigen & Hammes, 1963). This is an expected property for the multistep process of helix formation, which requires nucleation following the initial encounter of the two strands (see Discussion).

Site-Specific Hydrolysis of Substrate. The rate constant for dissociation of substrate from E·S was determined by making use of a hydrolysis reaction catalyzed by the ribozyme in the absence of G. Because this reaction had not been described before, it was characterized as follows. The 5′-end-labeled product of the reaction comigrates with p*G_2CCCUCU, the product of the G-dependent reaction, in denaturing PAGE (see Materials and Methods). This hydrolysis reaction was also observed to give the same 5′ product from substrates having A and AGU replacing the A_5 portion of G_2CCCUCUA$_5$ (T. McConnell and D. Herschlag, unpublished results). The 3′ product of the reaction with the A_5 substrate was not identified. The 3′ product from the

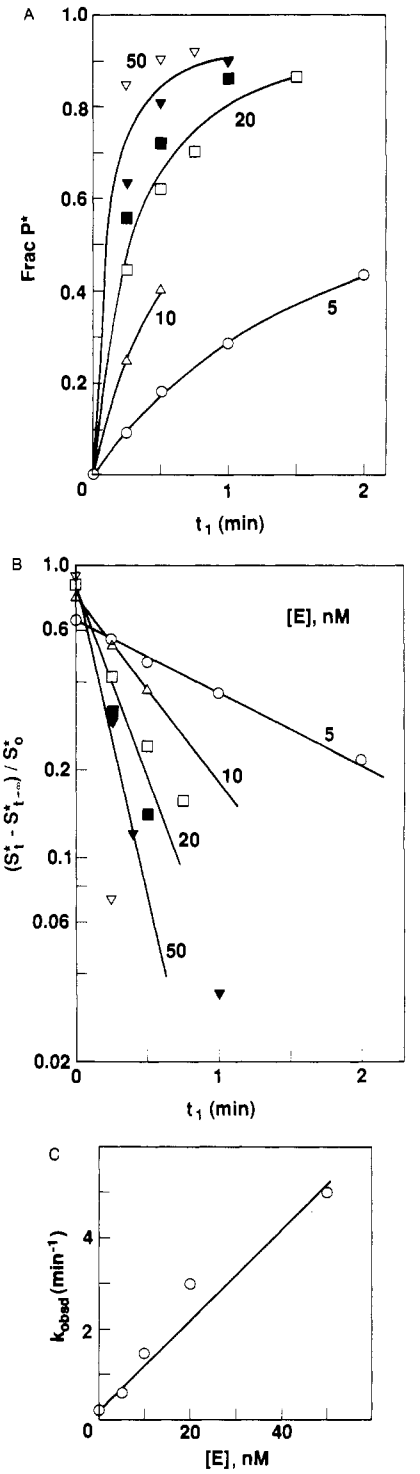


FIGURE 2: Determination of the rate of substrate binding from pulse–chase experiments. (A) Fraction of S* that forms P* (Frac P*) after preincubation of S* for time t_1 with 5 (○), 10 (△), 20 (□), and 50 (▽) nM E followed by a "chase" with 500 μM GTP and 2–3 μM unlabeled S or P. Open and closed symbols represent data from independent experiments. The amount of P* formed was constant for 0.25–3 min after the chase. (B) Semilogarithmic plot of the data from (A). The extent of reaction is given by $(S^*_t - S^*_{t\to\infty})/S^*_0$; S^*_t is the amount of S* remaining at time t; $S^*_{t\to\infty}$ is the amount of S* at the end point; and S^*_0 is the initial amount of S*. End points were calculated from the concentration of E and K_d(E·S) = 2 nM (see text). (C) Plot of the observed rate constants from (B) against the concentration of E; the point at [E] = 0 is from an independent pulse–chase experiment described in the text.

site-specific hydrolysis of the related substrate, G_2CCCUCUAGUp*Cp, was observed to migrate faster than the product from the reaction with G, GAGUp*Cp; com-

parison with standards was consistent with the formation of the expected product, pAGUp*Cp (J. Piccirilli and T. R. Cech, unpublished results). This rules out the possibility of reaction with a small amount of contaminating G.

Kinetic parameters for the site-specific hydrolysis reaction were determined by steady-state and single-turnover kinetic experiments analogous to those described above. The following values were obtained: $k_{cat}/K_m = 6 \times 10^7$ M^{-1} min^{-1}; k_{cat} = 0.05 min^{-1}; and k(single turnover) = $k_c(-G)$ = 0.7 min^{-1} (Table I; data not shown). The value of k_{cat} for this reaction is the same, within error, as that for the G-dependent reaction, suggesting that the rate-limiting step is the same (i.e., dissociation of product; see below). The value of k_{cat}/K_m is only slightly smaller than that for the G-dependent reaction, suggesting that, with subsaturating substrate, the rate-limiting step of the hydrolysis reaction is largely binding of substrate. This is shown directly in the next section.

Two pathways for the hydrolysis reaction were considered: (1) direct nucleophilic attack by water (or hydroxide ion) and (2) a two-step mechanism in which the 3′-terminal nucleotide of the ribozyme first attacks to give a covalently bound intermediate followed by hydrolysis to regenerate the ribozyme. There is precedence for each mechanism. The first is analogous to hydrolysis at the 5′-splice site in precursor rRNA; the occurrence of a small amount of splicing in the absence of added G can be explained by such hydrolysis followed by the normal second step of splicing, attack of the 5′-exon at the 3′-splice site (Inoue et al., 1986). An analogous hydrolysis reaction of the IVS, which gives cleavage at a site 15 nucleotides from the 5′-end that is homologous to the 5′-splice site, has been observed for IVS that has been β-eliminated to remove the reactive 3′-terminal G_{414} (Tanner & Cech, 1987). In mechanism 2, the first step is analogous to nucleotidyl-transfer reactions catalyzed by the L-19 IVS RNA and to the reverse of the second step of splicing; in these reactions the 3′-terminal G of the IVS acts as a nucleophile toward oligonucleotide substrates (Zaug & Cech, 1986; Woodson & Cech, 1989). The ribozyme used in our studies is made by transcription with T7 RNA polymerase, which can add one or more nucleotides beyond the end of the template strand (Milligan et al., 1987), so that there will be some 3′-terminal guanosine residues. The second step of mechanism 2 is a hydrolysis reaction, analogous to the intra- and intermolecular hydrolysis of sequences of the type GpN that has been observed in several reactions (Zaug et al., 1984; Inoue et al., 1986; Kay & Inoue, 1987).

The following observations support the first mechanism, a direct attack by solvent. (1) With saturating substrate, there is a burst of product formation that is nearly stoichiometric with E (data not shown). This shows that all of the ribozyme is active in the G-independent reaction, not just the 10–25% of the ribozyme molecules that contain a 3′-terminal G residue. In two preparations of L-21 *Sca*I ribozyme only ~10% of the ribozyme molecules contained a 3′-terminal guanosine residue (Zaug et al., 1988); the upper limit of 25% 3′-terminal G residues is obtained with the assumption that the 3′-terminal nucleotide is added randomly. (2) There is trapping of 70–80% of E·S* as P* in pulse–chase experiments without added G (see the following section); a maximum of ~10–25% trapping would be expected for a reaction that involved a 3′-terminal guanosine residue. (3) If the 3′-terminal G residue were involved in the hydrolysis reaction, then most of the substrate would have to dissociate from an initial E·S complex before reacting. This would result in an observed rate of reaction that is less than the rate of substrate dissociation for mechanism 2 with a covalent intermediate. However, the rate constant for a single turnover of the G-independent reaction, $k_c(-G)$ = 0.7 min^{-1} (saturating E; Table I), is larger than the rate of dissociation of substrate of 0.2 min^{-1} (see next section). Furthermore, there is good first-order behavior for ~3 half-lives for the hydrolysis reaction with E in excess. Again, this would not be expected for a reaction that was restricted to the subpopulation of ribozyme molecules terminating in G. Rather, in a reaction involving the 3′-terminal G there would be a burst of product formation of 10–25% followed by slower product formation as the substrate slowly dissociated from ribozyme without a 3′-terminal G and rebound to ribozyme containing a 3′-terminal G.

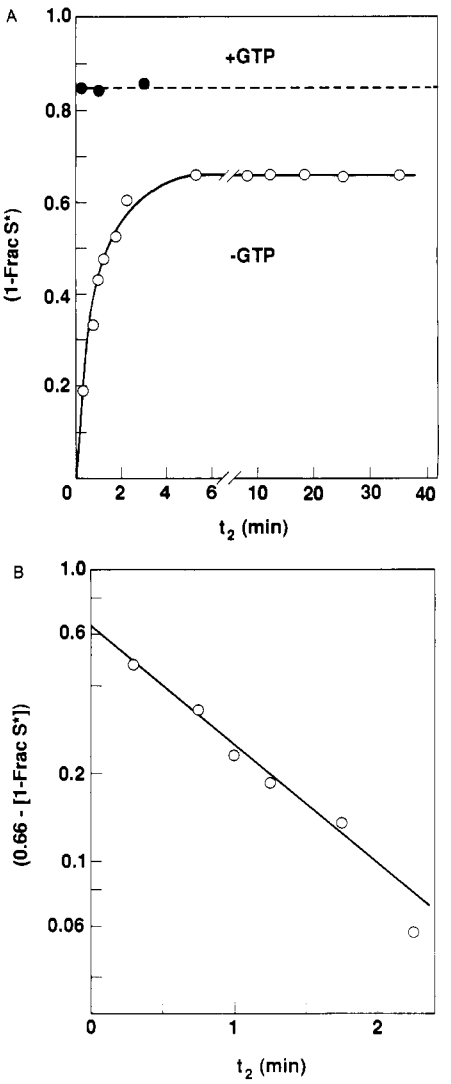
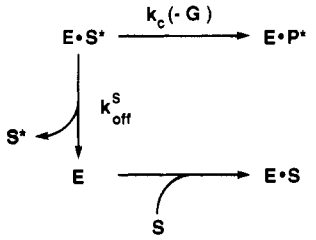


FIGURE 3: Determination of the rate of substrate dissociation from the extent and rate of trapping S* ($p^*G_2CCCUCUA_5$) as P* in pulse–chase experiments. (A) Ribozyme (50 nM) and S* were incubated for 0.25 min followed by a "chase" with (●) or without (○) 500 μM GTP. The disappearance of S* with time after the chase (t_2), 1 − Frac S*, is plotted; Frac S* is the fraction of S* remaining. (B) Semilogarithmic plot of the data from (A) for the disappearance of S* following the chase without GTP; the end point of 0.66 is the fraction of S* that forms product (part A).

Rate Constant for Dissociation of S from E·S. This rate constant, k_{off}^S, was determined according to Scheme IV from the partitioning of E·S* between dissociation and the hydrolysis of S described above. A pulse–chase experiment was carried out as described by Scheme III, except that G was omitted so that the hydrolysis reaction would occur during t_2. The solid line in Figure 3A shows the resulting slow formation of P*

Scheme IV



during t_2. The amount of labeled product formed is less than the amount formed when GTP is present (dashed line). Thus, some of the substrate dissociates over the time of the hydrolysis reaction (Scheme IV). The value of k_{off}^S can be determined from these data in two ways:

(1) Equation 2, which was derived from Scheme IV, relates the fraction of E·S* that proceeds to form P* [i.e., Frac(trapped)] to the rate constants for hydrolysis, $k_c(-G)$, and

$$\text{Frac(trapped)} = k_c(-G)/\{k_c(-G) + k_{off}^S\} \quad (2)$$

for dissociation of substrate, k_{off}^S. The fraction trapped is obtained from the amount of P* formed in the absence of G relative to that in the presence of G. This ratio from Figure 3A of Frac(trapped) = 0.66/0.85 = 0.78 and the independently measured $k_c(-G) = 0.7$ min^{-1} give $k_{off}^S = 0.2$ min^{-1} with use of eq 2.

(2) The second way to calculate k_{off}^S is from k_{obsd} and eq 3, which was also derived from Scheme IV. Figure 3B, a

$$k_{obsd} = k_c(-G) + k_{off}^S \quad (3)$$

first-order plot of the data of Figure 3A, gives $k_{obsd} = 0.9$ min^{-1}. This value and $k_c(-G) = 0.7$ min^{-1} give $k_{off}^S = 0.2$ min^{-1}, which is the same as the value obtained above from the product yield. Six similar experiments with 10–100 nM E gave k_{off}^S = 0.16–0.24 min^{-1} from the product yields (eq 2) and k_{off}^S = 0.19–0.25 min^{-1} from k_{obsd} (eq 3).

Equilibrium Dissociation Constant for E·S. The values of $k_{off}^S = 0.2 \pm 0.05$ min^{-1}, from the preceding section, and $k_{on}^S = (9 \pm 3) \times 10^7$ M^{-1} min^{-1}, from k_{cat}/K_m and direct measurements, give K_d(E·S) = 2 nM (range 1.3–4.2 nM). These values of k_{on}^S and k_{off}^S and the directly measured value of $k_c(-G) = 0.7 \pm 0.1$ min^{-1} can be used to calculate k_{cat}/K_m for the G-independent reaction from eq 4. The calculated value of $k_{cat}/K_m = (7 \pm 2) \times 10^7$ M^{-1} min^{-1} is the same, within error, as the observed value of 6×10^7 M^{-1} min^{-1}, supporting the validity of these measurements.

$$k_{cat}/K_m = k_{on}^S k_c(-G)/\{k_{off}^S + k_c(-G)\} \quad (4)$$

With Saturating Substrate, the Rate-Limiting Step Occurs after Chemistry. Figure 4A shows the time dependence of product formation in the reaction of 0.04–1.6 μM substrate with 0.5 mM GTP catalyzed by 7 nM E. The slopes of these lines are the same, within error, and give $k = 0.09$ min^{-1} after division by the concentration of E. The rate constant is independent of substrate concentration, so substrate is saturating and this rate constant corresponds to k_{cat} (Table I). Rate constants of 0.05–0.10 min^{-1} were obtained in several independent experiments (not shown).

The intercept of ~5 nM in Figure 4A is consistent with a burst that is stoichiometric with the 7 nM E. Such a burst is seen more clearly in Figure 4B, which shows product formation from 50 nM substrate with 10 nM E. The observed burst of 11 nM is stoichiometric, within error, with E. This stoichiometric burst shows that chemistry (and any steps preceding chemistry) occurs more rapidly than multiple turnover so that the rate-limiting step with saturating substrate

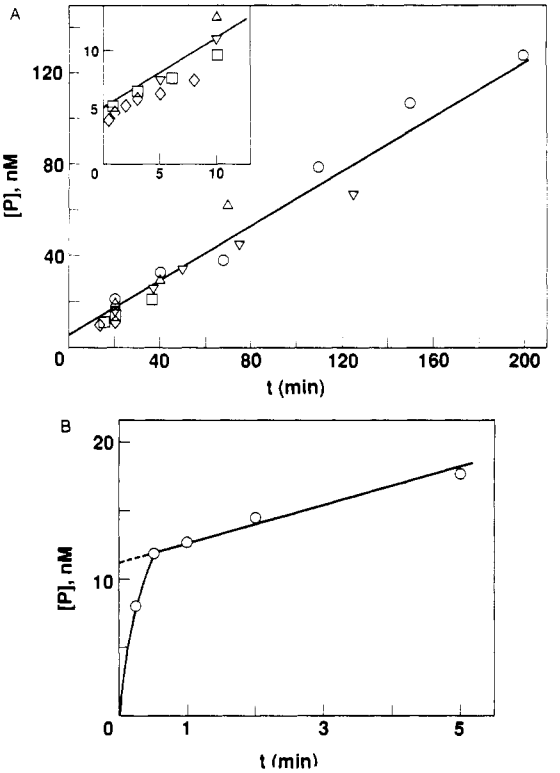


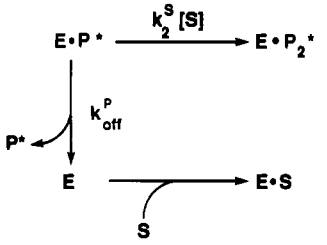
FIGURE 4: Time dependence of cleavage of p*G_2CCCUCUA$_5$ by the *Tetrahymena* ribozyme. (A) Reactions with 7 nM E, 500 μM GTP, and 0.04 (◇), 0.12 (□), 0.4 (▽), 0.8 (△), and 1.6 (○) μM S*. The inset shows the early time points. All data points correspond to <20% P* formation. (B) Reaction with 10 nM E, 50 nM S*, and 500 μM G. The final time point corresponds to 35% reaction.

occurs after chemistry. The rate constant $k = 0.14$ min^{-1} following the burst is consistent with the value of 0.1 min^{-1} obtained from Figure 4A. The stoichiometric burst shows that essentially all of the ribozyme is in an active conformation or in rapid equilibrium with an active conformation. The formation of a UV-induced intramolecular cross-link with nearly all (~80%) of the ribozyme molecules under these conditions is also consistent with a single population of molecules; the Mg^{2+} dependence of cross-link formation parallels that of ribozyme activity, suggesting that the cross-link reflects the active conformation (Downs & Cech, 1990; D. Celander and T. R. Cech, submitted for publication).

The rapid chemical step was further investigated in single-turnover experiments in which the concentration of E exceeded the concentration of S. The disappearance of substrate is first order, as determined by the linear plots of log [S] against time (data not shown). With 50 and 500 nM E and saturating G (500 μM) the rate constants for disappearance of substrate in a single turnover are ~3 and >4 min^{-1}, respectively, much greater than the value of $k_{cat} = 0.1$ min^{-1}. Thus k_{cat} represents a step that occurs after chemistry. The possibility that the small observed value of k_{cat} results from very strong product inhibition is ruled out by the observation that substrate and product bind with similar affinities (see Scheme I and below); in addition, there is no decrease in slope in Figure 4A as time increases and product accumulates, as would be expected from such strong product inhibition.

The Rate-Limiting Step with Saturating Substrate Is Dissociation of P, the 5′ Product Fragment. The results described above show that the rate-limiting step with saturating substrate (i.e., "k_{cat} conditions") occurs after the chemical conversion. This step could be dissociation of P, dissociation of GA_5, or a conformational change of the ternary E·P·GA_5

Scheme V



complex. Of course each dissociation could, in principle, involve more than one step. The following pulse–chase experiments give the rate constant for dissociation of P. This rate constant is equal to k_{cat}. Thus, dissociation of P is rate-limiting.

After E·S* reacts to form E·P*, there is a subsequent reaction that gives a product (P_2*) that migrates more slowly than the initial substrate in PAGE (not shown). This reaction is seen in pulse–chase experiments like that of Figure 3; this subsequent reaction is first order in unlabeled $pppG_2CCCUCUA_5$, which is used in the "chase". Nucleophilic attack by products (e.g., $G_2CCCUCU_{OH}$) after G residues of substrates that contain G at the 5′-end has been observed (eq 5; B. Flanegan, F. Murphy, and T. R. Cech,

$$\underset{(P^*)}{p^*G_2CCCUCU_{OH}} + \underset{(S)}{pppG_2CCCUCUA_5} \xrightarrow{E} p^*G_2CCCUCU(G)CCCUCUA_5 + pppG(G) \quad (5)$$
$$(P_2^*)$$

unpublished results). Presumably one of the 5′-terminal G residues occupies the G binding site of the ribozyme. Product P* then acts as a nucleophile in the reaction of eq 5, which resembles the reverse of the endonuclease reaction. The amount of P_2* formed decreases with increasing concentrations of GTP, as expected.

We have used this second reaction to determine the value of k_{off}^P, according to Scheme V. Figure 5 shows the results of two such experiments. Substrate was incubated with excess enzyme for 0.3 min to give E·S*, followed by addition of unlabeled S. The subsequent time courses with 0.8 and 3.2 μM unlabeled S are shown in Figure 5, panels A and B, respectively. Initially, S* is converted to P* via the site-specific hydrolysis reaction. Following a short lag for this conversion, a portion of the P* is then converted to the larger product, P_2*. With the higher concentration of unlabeled S in Figure 5B, the rate and extent of P_2* formation are greater than observed in Figure 5A. The amount of P* converted to P_2* depends on the rate of this reaction and the rate of P* dissociation from E, according to Scheme V. The value of k_{off}^P can be obtained from the fraction of P* that goes on to form P_2* and from the rate constant, k_{obsd}, for this conversion, according to eqs 6 and 7, which were derived from Scheme V. (Note that these equations are analogous to eqs 2 and 3 above.) For example, 0.54 of P* forms P_2* in Figure 5A, and

$$[P_2^*]_{t\to\infty}/([P^*]_{t\to\infty} + [P_2^*]_{t\to\infty}) = k_2^S[S]/(k_2^S[S] + k_{off}^P) \quad (6)$$

$$k_{obsd} = k_2^S[S] + k_{off}^P \quad (7)$$

k_{obsd} = 0.12 min^{-1} (first-order plot not shown). Eliminating k_2^S[S] from eqs 6 and 7, and then solving for k_{off}^P, gives k_{off}^P = 0.055 min^{-1}; a value of 0.062 min^{-1} is similarly obtained from Figure 5B. Values of k_{off}^P = 0.063 and 0.070 min^{-1} were obtained in analogous experiments with 1.6 μM unlabeled S and 50 and 100 nM E, respectively (data not shown). The value of k_2^S (Scheme V) obtained from these data with use of eqs 6 and 7 increases linearly with [S], so S is not saturating

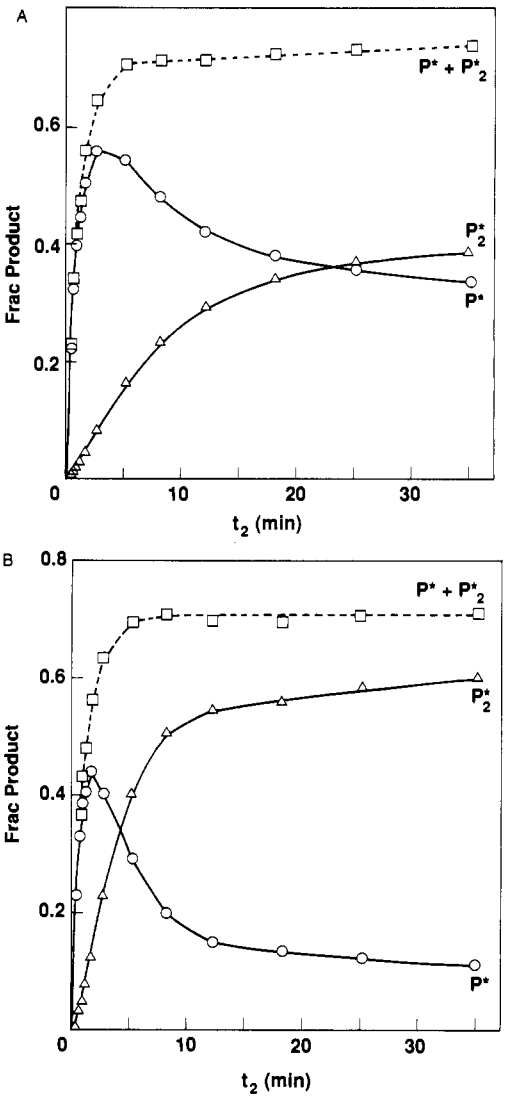


FIGURE 5: Determination of the rate of P* dissociation (P* = p*G_2CCCUCU) by monitoring the formation of a second product, P_2*, which is formed subsequent to P* in pulse–chase experiments. Ribozyme (100 nM) and S* (p*G_2CCCUCUA_5) were incubated for 0.33 min followed by a "chase" with 0.8 μM (A) or 3.2 μM (B) unlabeled S and *no* G. The formation of the normal product, P* (○), a second product, P_2* (△; see eq 5 and text), and the total of P* and P_2* (□; dashed line) with time after the chase are shown. Data are plotted as the fraction of S* that forms product (Frac Product).

the G binding site at 0.8–3.2 μM (not shown). Since the G site is not saturated, and k_{off}^P does not vary with the concentration of S, k_{off}^P is the rate constant for dissociation of P from the binary complex E·P.

The value of k_{off}^P = 0.06 min^{-1} is the same, within error, as the value of k_{cat} = 0.05–0.1 min^{-1} for both the G-dependent and the hydrolysis reactions (Table I). This strongly suggests that the dissociation of P (the 5′ fragment of the oligonucleotide substrate) is the rate-limiting step with saturating substrate and that this rate constant is not significantly affected by the presence of G. As described below, GA_5 dissociates before P, and the binding of P and G is essentially independent so that, with saturating G, dissociation of P will occur from the ternary complex E·P·G (Scheme I).

GA_5 Dissociates before P from the Ternary E·P·GA_5 Complex. Pulse–chase experiments analogous to those described in the previous section were carried out in the presence of 2.5 μM G in order to generate E·P*·GA_5; the amount of P_2* (eq 5) formed during the chase (t_2; Scheme III) with ~4 μM unlabeled substrate was compared to the amount formed from

E·P*, which was generated from reaction in the absence of G. Both E·P*·GA_5 and E·P* reacted during the chase to give end points with 70–80% of the P* converted to P_2*. (The low concentration of G and the 10-fold dilution upon addition of the "chase" with unlabeled S prevent significant inhibition or reversal of P_2* formation by G; for the control reaction in which E·P* was formed in the absence of G, G was added in the chase so that the concentration of G during the chase was the same in both reactions.) The formation of P_2* presumably involves binding of unlabeled S in the G binding site so that the formation of P_2* strongly suggests that GA_5 dissociates before P. This does not provide evidence for a compulsory order of dissociation, only for a faster dissociation of GA_5 than of P. There was no detectable lag in the conversion of P* to P_2* when P* was generated with G, so the dissociation of GA_5 is fast. A lower limit is $k_{off}^{GA5} \geq 1$ min^{-1} (data not shown).

Equilibrium Dissociation Constant for E·P and Rate Constant for P Binding to E. The dissociation constant for E·P (P = pppG_2CCCUCU) was determined from inhibition by 40–120 nM P of the reaction of 20–25 nM E, ~1 nM p*G_2CCC*G*CUA_5, a "mismatched" substrate, and 800 μM G. The reactions were followed for ≥60 min and were first order. The time expected to establish equilibrium between bound and free P is very fast relative to this time scale. For example, the rate constant for approach to equilibrium with 40 nM P is expected to be $k = k_{on}^{P}[P] + k_{off}^{P} \cong (10^8$ M^{-1} min^{-1}) (40 nM) + 0.06 ≅ 4 min^{-1}; this gives $t_{1/2} \cong 0.2$ min. Because the ribozyme concentration is well below saturation for the "mismatched" substrate, the inhibition is expected to give the value K_d(E·P) = K_i = 1 (±0.5) nM directly [see Scheme III of the following paper (Herschlag & Cech, 1990b)]. This value and k_{off}^{P} = 0.06–0.1 min^{-1} give $k_{on}^{P} = 10 \times 10^7$ M^{-1} min^{-1} [range (4–20 × 10^7 M^{-1} min^{-1}]. This is the same, within uncertainty, as the value $k_{on}^{S} = (9 \pm 3) \times 10^7$ M^{-1} min^{-1}.

Michaelis Constant for the Substrate $G_2CCCUCUA_5$. The value of K_m = 1 nM for S is obtained from division of k_{cat} = 0.1 min^{-1} by $k_{cat}/K_m = 9 \times 10^7$ M^{-1} min^{-1}. Since $k_{cat} = k_{off}^{P}$ and $k_{cat}/K_m = k_{on}^{S}$, K_m equals k_{off}^{P}/k_{on}^{S}. Thus, K_m is not equal to the dissociation constant for E·S. Nevertheless, the value of K_m is fortuitously similar to K_d(E·S) = 2 nM because of the similar binding of S and P.

Evidence for Independent Binding of $G_2CCCUCUA_5$ and G to the Ribozyme. The following data suggest that the binding of S and G to the ribozyme is random and independent; i.e., either S or G can bind first to the ribozyme, and there is no advantage to binding of S (or G) when G (or S) is bound (Scheme I).

Three possible mechanisms for binding are considered: (1) coupled binding, such that binding of S (or G) enhances the equilibrium binding of G (or S); (2) ordered binding, in which S (or G) must bind before G (or S) in order to form a competent ternary complex; and (3) random and independent binding.

The reaction of 10 nM E and ~1 nM S* was inhibited 30–40-fold by 40 nM P both in the presence and in the absence of 800 μM G (data not shown). Similarly, the binding constant of P is unaffected by the presence or absence of G in nondenaturing gels (Pyle et al., 1990). These results show that there is no significant coupling between the binding of product and G. An analogous absence of coupling for substrate and G is suggested by the similarity in binding of S and P and is supported by the following kinetic data. As shown above, the rate constant for binding of S is not significantly affected by G. The rate constant for dissociation of substrate could only be measured in the absence of G at pH 7.0 because the reaction with G is too fast to allow any significant dissociation of S in a pulse–chase experiment. Pulse–chase and steady-state experiments were therefore performed at pH 5.2, where the reaction is much slower; these data with the substrates $G_2CCCUCUA_5$ and $G_2CCCGCUA_5$ in the presence and absence of G are consistent with the absence of an effect of G on the value of k_{off}^{S} (Herschlag and Cech, unpublished results). (On the basis of estimates of the errors in rate and equilibria determinations, coupled binding that gives a small effect of ≤3-fold would not have been detected.)

The absence of thermodynamic coupling between S and G does not exclude kinetically ordered binding, in which either S or G must bind first due, for example, to a steric block created by initial binding of the other. The pulse–chase experiments described in the previous sections show that S can bind before G, that this bound substrate is competent to react, and that GA_5 can dissociate before P. The remaining pathway that would allow ordered binding would entail a block of the association and dissociation of S by G. The observation of the same value of k_{on}^{S} with and without G (see above) suggests that G does not provide such a kinetic block. In addition, the value of k_{cat}/K_m is the same with 100–2300 μM G (data not shown), concentrations both above and below the K_d(E·G) ≅ 0.5 mM (Herschlag & Cech, 1990b). Thus, the rate of substrate binding ($k_{on}^{S} = k_{cat}/K_m$; see above) is not decreased when G is bound to the ribozyme. Therefore, either G or S can bind first to the ribozyme; the binding is essentially random and independent.

The absence of coupling between G and the product shows that, during multiple-turnover reactions, the product will dissociate from either E·P·G or E·P, depending on the concentration of G present (Scheme I).

Rate Constant k_c for Reaction of the Ternary Complex. At sufficiently low concentrations of G, a step involving G will become rate-limiting. This could be the chemical conversion of the ternary complex (or an accompanying conformational change) or the binding of G. The following data suggest that it is the chemical conversion rather than binding of G that becomes rate-limiting at low concentrations of G and that the E·S·G ternary complex reacts with a calculated rate constant of $k_c \cong 350$ min^{-1} (Scheme I).

Rate constants for single-turnover reactions with 100 nM E, ~1 nM S*, and 0–3.5 μM G were determined. The observed linear increase in the rate constant with increasing concentration of G gives a second-order rate constant of (7 ± 1) × 10^5 M^{-1} min^{-1}. The high concentration of E was employed to ensure that binding of S is fast and quantitative; as expected, increasing the concentration of ribozyme 2.5-fold had no effect on the rate constant. The second-order rate constant is far below that for diffusion-controlled binding of 10^{10}–10^{11} M^{-1} min^{-1} (Eigen & Hammes, 1963), suggesting that the rate-limiting step is not binding of G. The rate constant for the rate-limiting step, k_c, is then determined from the second-order rate constant for reaction of E·S with G (see above) and the dissociation constant for E and G of $K_d \cong 0.5$ mM (Herschlag & Cech, 1990b): k_c = (7 × 10^5 M^{-1} min^{-1})(0.5 mM) = 350 min^{-1}. [The limits of uncertainty for K_d(E·G) of 0.3–1.1 mM give a range of values of k_c = 200–800 min^{-1}.]

The calculation assumes that the binding of G is not rate-limiting. It is conceivable that a conformational change that is required to allow the binding of G dramatically lowers the observed rate constant for binding relative to the diffusional limit. If the binding of G were rate-limiting, then the rate constant for conversion of the ternary complex, k_c, would be

greater than ~350 min^{-1}. This is because k_c would need to be faster than k_{off}^G in order not to be rate-limiting, and a value of k_{off}^G = 350 min^{-1} is obtained by assuming that the binding of G is rate-limiting with $k_{on}^G = 7 \times 10^5$ M^{-1} min^{-1} and K_d(E·G) = 0.5 mM (Herschlag and Cech, following paper in this issue).

There is an alternative mechanism to that with k_c representing the rate-limiting step for the central conversion (i.e., a chemical step, conformational step, or combination of these) that must be considered. A rapid conversion of the ternary substrate complex, E·S·G, to the ternary product complex, E·P·GA_5, followed by rate-limiting dissociation of GA_5 from the ternary complex would give k_c that represents the equilibrium between the ternary complexes and the rate constant for GA_5 dissociation according to eq 8. However, the following analysis shows that this mechanism is unlikely, as it would require binding of GA_5 to E·P to occur at ~10^6 M^{-1} min^{-1}, which is ~10^4-fold slower than diffusion-controlled binding. The overall equilibrium for the reaction free in solution is near 1, as expected for a simple transesterification reaction (Herschlag, T. McConnell, and Cech, unpublished observations), and the binding affinity of S and P are similar (see above). Therefore, the value of K_2 for the equilibrium E·S + G ⇌ E·P + GA_5 is ~1 (eq 9). The equilibrium

$$k_c = k_{off}^{GA5} K_3 \qquad K_3 = [E\cdot P\cdot GA_5]/[E\cdot S\cdot G] \quad (8)$$

represented by K_2 is related to the equilibrium for the ternary complexes (K_3; eq 8) by the dissociation constants for G and GA_5 according to eq 10. Combining eqs 10 and 8, with use of $K_d^{GA5} = k_{off}^{GA5}/k_{on}^{GA5}$, gives eq 11. Solving eq 11 for k_{on}^{GA5} with use of k_c = 350 min^{-1}, K_2 = 1, and K_d^G = 0.5 mM gives $k_{on}^{GA5} = 7 \times 10^5$ M^{-1} min^{-1}. The value of $K_d^G \cong 0.5$ mM, which was determined for dissociation from the binary E·G complex (Herschlag and Cech, following paper), is used in eq 11 for dissociation from the ternary complex because of the evidence that binding of S and G are independent (see above).

$$K_2 = K_3 K_d^{GA5}/K_d^G \qquad K_d^G = [E\cdot S][G]/[E\cdot S\cdot G]$$
$$K_d^{GA5} = [E\cdot P][GA_5]/[E\cdot P\cdot GA_5] \quad (10)$$

$$k_c = k_{off}^{GA5} K_2 K_d^G / K_d^{GA5} = k_{on}^{GA5} K_2 K_d^G \quad (11)$$

Thus, we adopt a model in which k_c represents a step or steps associated with the chemical conversion of the ternary complex, rather than the binding of G or the dissociation of GA_5.

Discussion

The ribozyme from *T. thermophila* pre-rRNA catalyzes the sequence-specific RNA endonuclease reaction shown in eq 1. The following is a brief description of the reaction proceeding from left to right in Scheme I.

Binding of guanosine and binding of the oligonucleotide substrate are independent and random; i.e., binding of one substrate does not significantly affect the rate or equilibrium for binding of the other substrate. The oligonucleotide substrate binds ~10^4-fold stronger than expected for simple helix formation with the 5′-exon binding site (Figure 1B; see below). The binding of G with $K_d \cong 0.5$ mM was determined from data in the following paper (Herschlag & Cech, 1990b).

After formation of the ternary E·S·G complex, reaction occurs rapidly with $k_c \cong 350$ min^{-1}. However, the chemical step does not enter into the steady-state rate expression with saturating G. With subsaturating oligonucleotide substrate the rate-limiting step is binding, as every time the substrate binds it reacts ($k_c \cong 350$ min^{-1}) rather than dissociates (k_{off}^S = 0.2 min^{-1}). This ribozyme can thus be described as having achieved catalytic perfection, because the only step limiting its second-order reaction is substrate binding (Alberty & Knowles, 1976). Substrate binding is occurring as fast as can be expected for helix formation (see below).

With saturating oligonucleotide substrate the rate-limiting step for the ribozyme reaction is the slow dissociation of the product that is analogous to the 5′-exon. This slow dissociation could ensure efficient self-splicing by preventing the release of the 5′-exon after the first step of splicing [see Herschlag and Cech (following paper)]. It should be realized that the *Tetrahymena* IVS has evolved to perform a single self-splicing event, not catalysis with multiple turnovers.

Equilibria for Binding of the Oligonucleotide Substrate and Product. The similar binding constants of substrate and product, with K_d = 2 and 1 nM, respectively, show that the pA_5 sequence of the substrate beyond the cleavage site makes no net contribution to binding. (The three U residues of the internal guide sequence that could potentially base pair with three A residues 3′ of the 5′-splice junction in the splicing reaction are not present in the L-21 *Sca*I ribozyme used herein.) The absence of stabilization from the pA_5 sequence is contrary to the increase in stability of simple RNA·RNA helices expected from addition of a dangling 3′A residue (Freier et al., 1986) and the presumed interaction of the active site with the reactive phosphoryl group in the transition state. The tertiary structure of the ribozyme (see below and following paper) could prevent the stacking interactions that provide the stabilization from addition of a terminal nucleotide. However, it remains surprising that there is no stabilization from addition of the reactive phosphoryl group. It is possible that this reactive phosphoryl group interacts favorably with the ribozyme in the transition state but not in the ground state or that a positive interaction is offset by destabilization from the A_5 moiety.

The dissociation constants of 1 and 2 nM for the oligonucleotide product and substrate, respectively, reflect ~10^4-fold (~6 kcal/mol) stronger binding than calculated for base pairing between the oligonucleotide and GGAGGG, the 5′-exon binding site, at 50 °C (Figure 1B; Freier et al., 1986; D. Turner, personal communication). This additional binding energy strongly suggests that tertiary interactions of the ribozyme with the oligonucleotide contribute to binding (see also Herschlag and Cech, following paper). Determination of the binding constant of a fluorescently labeled oligonucleotide to a circular form of the IVS by fluorescence quenching has also led to the conclusion that binding is stronger than expected for simple helices in solution (Sugimoto et al., 1989). Similar conclusions have been reached from steady-state kinetic studies of circle-opening reactions and the assumption that K_m equals K_d (Sullivan & Cech, 1985; Sugimoto et al., 1988).

Rate of Binding of the Oligonucleotide Substrate and Product. The rate constants for binding of the oligonucleotide substrates and products of 10^7–10^8 M^{-1} min^{-1} (Scheme I and Herschlag and Cech, following paper) are far below the diffusional limit of ~10^{11} M^{-1} min^{-1} for collision of small molecules (Eigen & Hammes, 1963). However, these observed binding rates are similar to the observed rate constants of ~10^7–10^9 M^{-1} min^{-1} for helix formation between two oligonucleotides (Porschke & Eigen, 1971; Craig et al., 1971; Porschke et al., 1973; Ravetch et al., 1974; Breslauer & Bina-Stein, 1977; Nelson & Tinoco, 1982). Most diffusional collisions between oligonucleotides are thought to be nonproductive because the "nucleation" of two or three base pairs is required for formation of the final bound species to be faster

than dissociation, so that helix formation is slowed relative to diffusional encounter [e.g., Porshke and Eigen (1971)].

The "matched" substrate and product bind slightly faster than their "mismatched" counterparts, which contain a mismatch in the helix with the 5′-exon binding site (Scheme I and Herschlag and Cech, following paper). This small difference in binding rates is consistent with the "nucleation" model for helix formation, as nucleation would be less probable for the substrate containing a mismatch.

The additional energy of binding of oligonucleotides and the ribozyme from tertiary interaction (see above) does not result in binding that is faster than helix formation in model systems. There would be no effect on the rate of binding if, for example, the additional binding energy were not realized until after helix formation, either because of a required order for the "microscopic" binding, perhaps with a conformational change that positions the tertiary groups, or because it is only the loss in entropy upon helix formation that renders the additional interaction energetically favorable (Jencks, 1981).

The hammerhead and hairpin ribozymes, which also recognize their substrates by base pairing, exhibit values of $k_{cat}/K_m \cong 10^7$ M^{-1} min^{-1} (Hampel & Tritz, 1989; Fedor & Uhlenbeck, 1990; Hampel et al., 1990). It is possible that helix formation is also the rate-limiting step for these ribozymes with subsaturating substrate. Substrate recognition by RNase P, unlike the other ribozymes, does not appear to occur via simple helix formation. For the reaction of the RNA component of RNase P at very high salt (2 M NH_4Cl, 0.1 M $MgCl_2$), the observed burst of product formation suggests that the dissociation of product is rate-limiting (Reich et al., 1988). The interactions of this ribozyme with the substrate and large product fragment (i.e., the tRNA product) are thought to be similar (Altman, 1989; Pace & Smith, 1990), making it reasonable that substrate dissociation occurs at a rate that is similar to the rate of product dissociation and slower than the rate of the chemical step. If substrate dissociation is indeed slower than chemistry, then binding is rate-limiting for the reaction at subsaturating substrate; this can be understood by realizing that the free energy barrier for substrate dissociation, and therefore substrate binding, is larger than the barrier for the chemical step so that the transition state for binding is the highest barrier in the free energy profile.

These ribozymes can be described as having achieved or nearly achieved "evolutionary perfection", as defined by Albery and Knowles (1976), because they react at or near the rate of binding. Enzymes can be considered "perfect" when diffusive steps limit the rate so that an increase in the rate of the chemical step does not increase the observed catalysis. It should be realized that the rate of binding of substrate to the *Tetrahymena* ribozyme, and of helix formation in general, is less than the rate of diffusional encounter because of the requirement for additional rearrangement to provide nucleation. The requirement for nucleation presumably limits the rate constant for a "perfect" ribozyme that uses base pairing for recognition to a value significantly lower than that for diffusional encounter. In contrast, several aminoacyl-tRNA synthetases bind tRNA substrates $\sim 10^2$-fold faster than the *Tetrahymena* ribozyme binds its RNA substrate (Fersht, 1985). Factors that affect the rate constant for binding of substrates to protein enzymes are described below.

Another criterion for a good enzyme is that the value of K_m be greater than the concentration of the substrate in vivo so that bound forms of the enzyme do not accumulate and the enzyme remains free to catalyze successive turnovers of the substrate (Fersht, 1974; Jencks, 1975). However, this criterion cannot be applied to the ribozymes because the biological role of all of these ribozymes, save RNase P, appears to involve a single intramolecular catalytic event. In fact, several small mutational changes in the *Tetrahymena* ribozyme increase K_m without decreasing k_{cat}/K_m so that selective pressure for multiple turnover in vivo would be expected to produce a "better" catalytic ribozyme (B. Young, Herschlag, and Cech, in preparation).

It is of interest to compare the factors that affect rates of binding to protein and RNA enzymes. Protein enzymes also bind substrates at rates below the diffusional limit for small molecules of 10^{11} M^{-1} min^{-1}, with typical values of 10^8–10^{10} M^{-1} min^{-1} (Hammes, 1982; Fersht, 1985). At least some of this rate decrease presumably arises from the small size and limited accessibility of the binding site [e.g., Schmitz and Schurr (1972) and McCammon and Northrup (1981)]. Indeed, enzymatic binding sites are routinely in crevices, and flaps or hinges that close to surround a substrate can limit access to the active site [e.g., see Miller et al. (1980) and references in Herschlag (1988)]. Cationic inhibitors of acetylcholinesterase bind $\sim$10-fold faster than the substrate, acetylcholine, presumably because the inhibitors have fewer steric restrictions to binding (Rosenberry & Neumann, 1977; Jencks, 1980). X-ray crystallographic structures of myoglobin suggest that the heme is occluded from solvent so that a series of conformational changes are required for ligand binding and dissociation (Case & Karplus, 1979; Debrunner & Frauenfelder, 1982; Johnson et al., 1989). Multiple steps may lower the rate of binding to proteins, as it does for nucleic acid helix formation (Burgen et al., 1975). For example, it is unlikely that the nicotinamide and adenine moieties of NAD find their binding sites on a dehydrogenase simultaneously; a rate of dissociation of the partially bound species that is greater than the rate at which the unbound portion finds its site would slow the observed rate of binding relative to diffusional encounter. Alternate conformations of proteins and nucleic acids and their ligands could also lower rates of binding.

An additional mechanism that could decrease rates of binding is the requirement to desolvate a protein's active site and its substrate [e.g., Bartlett and Marlowe (1987) and Holden et al. (1987)]. Trapping of highly reactive carbocations by amines and carboxylate ions occurs at rates $\sim$10-fold below the diffusional limit, apparently due in large part to the requirement to desolvate these nucleophiles (Richard & Jencks, 1984; Richard, 1987). The requirement to desolvate several such groups in an enzyme active site could provide a significant barrier to binding. Furthermore, the presence of a number of hydrogen-bonding groups at an enzyme's active site could cause a single water molecule to have multiple hydrogen bonds to groups that are geometrically fixed, thereby creating a larger barrier to desolvation than that observed with small molecules.

In summary, the *Tetrahymena* ribozyme and some other ribozymes bind substrates at rates approaching those for protein enzymes. The multistep process of helix formation that presumably occurs in substrate binding to these ribozymes may be analogous to substrate binding to protein enzymes involving nucleation with sequential binding of portions of large substrates. Sequential binding of portions of a substrate to an enzyme might be required to allow for multiple desolvation events.

Does Rate Constant k_c Represent the Chemical Step? The simplest interpretation of our data is that the chemical conversion of the ternary complex occurs with the rate constant $k_c \cong 350$ min^{-1} (Scheme I). However, if the binding of G were

slower than diffusion controlled by more than 10^4-fold, so this binding were rate-limiting for $(k_{cat}/K_m)^G$ (see Results), then k_c would be greater than 350 min^{-1}. Furthermore, the involvement of a conformational step cannot be excluded, as is generally the case. An extreme model would entail a conformational change of the ternary complex with $k_c = 350$ min^{-1} that is essentially irreversible, followed by a slower chemical conversion. The observed rate constant for formation of product in single-turnover experiments of >4 min^{-1} (see Results) provides an absolute lower limit for the rate constant of the chemical step and of any conformational steps, regardless of the proposed mechanism.

An earlier model involving a rate-limiting conformational change in the ribozyme reaction was based on the observation that a single-turnover reaction with a "mismatched" substrate was faster than that with the "matched" substrate studied herein (Zaug et al., 1988). However, after preincubation with Mg^{2+} to activate the ribozyme, the matched substrate reacts faster than the mismatched substrate under single-turnover conditions, so there is no need to invoke a conformational change (Herschlag and Cech, following paper). The previous data appear to result from the slow initial folding of the ribozyme into an active conformation upon addition of Mg^{2+} and are thus not pertinent to the reaction scheme of the fully folded ribozyme (see below). The absence of a significant rate decrease upon substitution of one of the phosphoryl oxygen atoms at the cleavage site by sulfur (McSwiggen & Cech, 1989) is now interpreted to be consistent with the evidence herein for rate-limiting substrate binding and product release (Herschlag, J. Piccirilli, and Cech, in preparation). Although a large intrinsic thio effect would have been expected to result in a significant rate decrease with the thio-substituted mismatched substrate, $G_2CCCGCUAGU$, because binding and chemistry are each partially rate-limiting (Herschlag and Cech, following paper), studies of phosphate diesters in nonenzymatic reactions reveal that the intrinsic thio effect is modest (Herschlag, J. Piccirilli, and Cech, in preparation).

Again, we suggest the simplest working hypothesis, in which k_c represents the rate of the chemical transformation in the ribozyme reaction. We are aware of no data that warrant proposal of a more complex mechanism.

The calculated rate constant of $k_c \cong 350$ min^{-1} for the chemical step represents a $\sim 10^{11}$-fold rate advantage over the estimated rate of hydrolysis in solution, comparable to rate advantages achieved by protein enzymes. The solution rate of $k_{uncat} = 3 \times 10^{-9}$ min^{-1} was estimated as follows: The rate constant for reaction of water with dimethyl phosphate monoanion was estimated from the rate constant for reaction of hydroxide ion, $k_{OH} = 8 \times 10^{-9}$ M^{-1} min^{-1} (attack at phosphorus; 50 °C; Kumamoto et al., 1956; Haake & Westheimer, 1961), the linear free energy relationship $\beta_{nuc} = \delta(\log k)/\delta pK_{nuc} \cong 0.3$ (Kirby & Younas, 1970), and the concentration of water of 55 M with use of eq 12. This rate constant of $k_w = 3 \times 10^{-12}$ min^{-1} was then adjusted according to eq 13 with use of the linear free energy relationship $\beta_{lg} = \delta(\log k)/\delta pK_{lg} \cong -1$ (Kirby & Younas, 1970) to account for the pK_a of the ribosyl anion leaving group being ~3 lower than that of methoxide ion, the leaving group in the hydrolysis of dimethyl phosphate. It should be noted that k_{uncat} was calculated for the intermolecular attack by water, which is analogous to the intermolecular attack by guanosine in the ribozyme reaction. This reaction is much slower than the observed intramolecular solution reaction of RNA that gives formation of a 2',3'-cyclic phosphate product.

$$k_w = (55\ M)(k_{OH})\ 10\ \exp\{(\beta_{nuc})(pK_a^{HOH} - pK_a^{HO^-})\} \quad (12)$$

$$k_{uncat} = (k_w)\ 10\ \exp\{(\beta_{lg})(pK_{ribose} - pK_{CH3OH})\} \quad (13)$$

What Limits the Rate of Self-Splicing? The large value of $k_c \cong 350$ min^{-1} at 50 °C compared with the maximal rate of self-splicing of ~1 min^{-1} at 42 °C (Williamson et al., 1987) introduces the possibility that a conformational step slows self-splicing. A large rate difference is also obtained from comparisons of k_c for the endonuclease reaction with the maximal rate of splicing both at 30 °C (Herschlag and Cech, unpublished results).

Hydrolysis Reaction. The site-specific hydrolysis reaction that occurs in the absence of added guanosine is ~500-fold slower than the reaction of the ternary complex with guanosine bound [$k_c/k_c(-G) = (350\ min^{-1})/(0.7\ min^{-1}) = 500$]. This suggests that interactions of the ribozyme with the guanosine away from the nucleophilic 3'-hydroxyl group account for ~4 kcal/mol of the ~16 kcal/mol of total transition-state stabilization by the ribozyme. [The value of 16 kcal/mol is obtained from the rate enhancement of k_c/k_{uncat} of $\sim 10^{11}$-fold described above and the equation $\Delta G° = -RT \ln (k_c/k_{uncat})$.] In addition, this relatively small advantage for reaction with guanosine compared to solvent suggests that either water or hydroxide can be activated for nucleophilic attack by the ribozyme or that most of the catalytic power of the ribozyme derives from interactions with the phosphoryl group and the oligonucleotide leaving group.

Explanation of Earlier Results. The value of $k_{cat} = 0.1$ min^{-1} (Table I) is similar to the previously reported value of $k_{cat} = 0.04$ min^{-1} (Zaug et al., 1988). However, the value of $K_m = 1$ nM obtained herein is much different from the previous value of $K_m = 160$ nM. It is likely that folding of the ribozyme affected the earlier steady-state measurements, because the reactions were initiated by the addition of $MgCl_2$ to a denatured ribozyme in EDTA. Preliminary experiments have suggested that preincubation with Mg^{2+} does indeed cause a slow conformational change of the ribozyme to a more active form, so ribozyme was preincubated with Mg^{2+} in the present work (Materials and Methods and Herschlag, P. Legault, and Cech, unpublished results).

Problems in the earlier steady-state investigation may have been obscured by the use of an experimental protocol that is not generally valid for obtaining steady-state kinetic parameters. The earlier determination was performed with 10 nM ribozyme and 100–4000 nM substrate, and reactions were followed over the first 10% of the reaction. The error in this methodology is that only the first turnover of the ribozyme is followed at the low substrate concentration, whereas at higher substrate concentrations predominantly the subsequent turnovers are monitored. If the initial turnover is fast and a subsequent step is slow, then apparent saturation behavior can be observed with this experimental setup.

Limited steady-state analysis with the related substrate, $G_2CCCUCUAGU$, gave values of $k_{cat} = 0.13$ min^{-1} and $K_m = 70$ nM (McSwiggen & Cech, 1989), which are similar to and much larger than the respective values for $G_2CCCUCUA_5$ obtained herein. However, the previous kinetic data were obtained over a 10-fold range of substrate concentration, but the observed rate of reaction varied only ~2-fold. Thus, the value of k_{cat} should be rather accurate, but the value of K_m is not expected to be accurate. The values of k_{cat}/K_m and $K_d(E{\cdot}S)$ are the same for the $G_2CCCUCUAGU$ and $G_2CCCUCUA_5$ substrates (Herschlag, J. Piccirilli, and Cech, in preparation).

In contrast to the problems in earlier determinations of steady-state kinetic parameters with the "matched" substrate,

the values of k_{cat} = 6 min^{-1} and K_m = 0.3 μM obtained for the "mismatched" substrate, $G_2CCCGCUA_5$, in the following paper (Herschlag & Cech, 1990b) are similar to the previously reported values of k_{cat} = 1–3 min^{-1} and K_m = 0.3–0.7 μM for this and the related substrate, $G_2CCCGCUAGU$ (Zaug et al., 1988; McSwiggen & Cech, 1989). The weaker binding of the mismatched substrate (K_d = 2.5 μM) presumably prevented a burst of product formation with low concentrations of substrate that could otherwise have interfered with the steady-state analysis, as described above.

Conclusions and Future Prospects

On the basis of the experiments described here, we have developed the following view of the cleavage of a "matched" RNA substrate catalyzed by the L-21 *Sca*I *Tetrahymena* ribozyme (pH 7, 10 mM Mg^{2+}, 50 °C): (1) The two substrates, RNA and guanosine, bind randomly and independently to the ribozyme. We suggest a physical picture in which independent portions of the RNA structure bind each substrate. (2) The RNA substrate binds very tightly (K_d = 2 nM) due to an extraordinarily slow off-rate of 0.2 min^{-1}. Tertiary interactions provide 6 kcal/mol binding energy beyond that derived from the complementary base pairing interactions [see also Sugimoto et al. (1989); Herschlag and Cech, following paper; and Pyle et al. (1990)]. (3) Once both substrates are bound, they react very quickly. The calculated rate constant of 350 min^{-1} leads to a new appreciation of the catalytic power that is possible with an active site composed of RNA. This corresponds to a rate enhancement of $\sim 10^{11}$ over an estimated rate constant for hydrolysis. Because the reaction is limited by a diffusive step (binding of the substrate RNA via base pairing), any "improvement" of active site groups would fail to increase the overall reaction rate; by this criterion, the ribozyme has attained catalytic perfection (Albery & Knowles, 1976). (4) The RNA product that contains the newly added guanosine at its 5′-end exits the active site rapidly. The other RNA product, which is base-paired to the ribozyme active site, is released very slowly (k_{off}^P = 0.1 min^{-1}). Dissociation of this product is rate-limiting under saturating substrate conditions. A corollary is that any mutation or altered reaction condition that increases the turnover number must necessarily increase product dissociation. (5) The site-specific hydrolysis reaction is ~500-fold slower than the reaction with guanosine. This suggests that interactions with the guanine and ribose moieties, away from the nucleophilic 3′-hydroxyl, provide ~4 kcal/mol of transition-state stabilization. The rest of the ~16 kcal/mol of transition-state stabilization presumably arises from activation of the 3′-hydroxyl and/or interactions with the phosphoryl group and the oligonucleotide leaving group.

Approximately 300 site-specific mutations of the *Tetrahymena* intervening sequence have been tested for their effect on RNA splicing or on cleavage at the 5′-splice site, and additional mutations have been studied in other group I intervening sequences [e.g., Waring et al. (1985), Burke et al., (1986), Been et al. (1987), Belfort et al. (1987), Flor et al. (1989), Michel et al. (1989), and Couture et al. (1990)]. The phenotypes of such mutants have in some cases been interpreted in terms of K_m and k_{cat} values, steady-state kinetic parameters that have not yet been defined in terms of elemental rate constants. Indeed, the large rate of the chemical step obtained in this study relative to k_{cat} values for self-splicing has raised the possibility that a conformational step that is absent or not rate-limiting in the ribozyme-catalyzed cleavage of oligonucleotides slows the self-splicing reaction. It should now be possible, with use of the type of analysis described herein, to analyze separately the effects of mutations on K_d for the RNA substrate (stability of the P1 helix), K_d for guanosine (see Herschlag and Cech, following paper), and the chemical reaction between these two substrates. This type of analysis has already been applied to cleavage of a DNA substrate (Herschlag & Cech, 1990a) and a "mismatched" RNA substrate (Herschlag and Cech, following paper).

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