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: \( G_2CCCUCUA_5 + G \rightarrow G_2CCCUCU + GA_3 \) (\( G = \) 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 \( k_{\text{cat}}/K_m = 9 \times 10^7 \text{ M}^{-1} \text{ min}^{-1} \) 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 \( k_{\text{cat}} = 0.1 \text{ min}^{-1} \) represents rate-limiting dissociation of the 5'-exon analogue, \( G_2CCCUCU \). The product \( GA_3 \) dissociates first from the ribozyme, \( G_2CCCUCU \), and the substrate, \( G_2CCCUCUA_5 \), to the 5'-exon binding site of the ribozyme, with \( k_{a_2} = 1 \text{ to } 2 \text{ nM} \), shows that the \( a_2 \) portion of the substrate makes no net contribution to binding. Both the substrate and product bind \( \sim 10^4 \text{-fold} \) (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 \( k_c \approx 350 \text{ min}^{-1} \), 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, \( k_c(-G) = 0.7 \text{ min}^{-1} \), is \( \sim 500 \text{-fold} \) slower than that with saturating guanosine. The value of \( k_{\text{cat}}/K_m = 6 \times 10^7 \text{ M}^{-1} \text{ min}^{-1} \) 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 \( k_{\text{cat}}/K_m \) for protein enzymes, can be considered to have achieved “catalytic perfection” [Albery, W. J., & Knowles, J. R. (1976) *Biochemistry* 15, 5631-5640].

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L-21 ScaI Ribozyme. This ribozyme was prepared essentially as described previously (Zaug et al., 1988). Briefly, plasmid pT7L-21 was cut with ScaI restriction endonuclease, ethanol precipitated, and resuspended in H2O. 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 \( e_{260} = 3.2 \times 10^6 \text{M}^{-1} \text{cm}^{-1} \) (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 H2O 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 with treatment with calf alkaline phosphatase followed by polynucleotide kinase and \([\gamma-32P]ATP\), 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 MgCl₂. Initial experiments revealed increased rates of reaction when the ribozyme was preincubated with 10 mM MgCl₂ 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, MgCl₂, 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.
Ribozyme Catalysis

Scheme I

during reactions over several hours.

Oligonucleotides used in these reactions that were 32P-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, MgCl2, 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 MgCl2, 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 chase 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, G2CCCUCUA3, 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 Scal RNA, is referred to as "E" or "ribozyme". The oligonucleotide product, G2CCCUCU, is referred to as "product" or "P*".

Determination of kcat/Km for G2CCCUCUA3. The second-order rate constant for reaction of E-G and S (kcat/Km) was determined in single-turnover experiments with saturating GTP. The value of kobs was increased linearly with the concentration of E (7–50 nM E, 500 μM GTP). A value of kcat/Km = (9 ± 3) × 107 M−1 min−1 was obtained from kobs divided by [E] (Table I). Replacement of G for GTP had no effect on the value of kcat/Km. Less precise determination of these parameters by initial rate measurements in multiple-turnover experiments with 0.2 mM E, 0.3–40 mM S, and 500 μM GTP gave values of kcat/Km = 5 × 107 M−1 min−1 and kcat = 0.1 min−1 from a reciprocal plot (data not shown), in reasonable agreement with values determined above and below.

The Rate-Limiting Step for kcat/Km Is Substrate Binding. Pulse–chase experiments were carried out to investigate the partitioning of the ternary complex E-S-G between reaction and dissociation of S (Scheme II; Rose et al., 1974). As outlined in Scheme III, an excess of E was mixed with 32P-labeled substrate (S*) for time t1 to allow formation of the E-S* complex. G and an excess of unlabeled substrate 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 t1, and no correction was made for the 2–5% of the S* that is typically unreactive.

These data show that the chemical step (kcat) is much faster than dissociation of S* from the ternary complex, E-S*-G.
Table II: Partitioning of E-S*-G (S* = pG2CCCUCUAGp) between Reaction To Form P* and Dissociation of S* in "Pulse-Chase" Experiments

<table>
<thead>
<tr>
<th>[E] (nM)</th>
<th>t1 (min)</th>
<th>fraction of S* trapped as P* after <em>chase</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.25</td>
<td>0.88</td>
</tr>
<tr>
<td>100</td>
<td>0.50</td>
<td>0.89</td>
</tr>
<tr>
<td>100</td>
<td>0.33</td>
<td>0.88</td>
</tr>
<tr>
<td>200</td>
<td>0.33</td>
<td>0.90</td>
</tr>
</tbody>
</table>

*Experimental protocol described in the text and in Scheme III.
+The amount of S* that formed P* during t1 (10–30%, depending on the duration of t1) was determined in control reactions and subtracted from the total formation of P* prior to determining the fractions listed in this column. The reaction during t1 in the absence of G arises from hydrolysis (see text). The amount of P* formed was constant over t1 (Scheme III) from 0.25 to 4 min. +With 500 μM GTP. +With 1 mM G.

(koffS; Scheme II). Since essentially every time substrate binds it will go on to react, the rate-limiting step under kcat/Km 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*, t1, was varied, and the amount of E-S* formed in t1 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 kobsd = koffS[E] + kcatS. Figure 2B shows the first-order plots that were used to obtain koffb. The end points, which were calculated from the concentration of E and Kd(E-S) = 2 nM (see Scheme I and below), give good straight lines in Figure 2B. A value of koffS = 10 × 10⁷ M⁻¹ min⁻¹ is obtained from the slope of the plot of koffb against [E] in Figure 2C. The intercept at [E] = 0 is equal to koffS 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 koffS determined in this experiment is the same, within error, as the value of kcat/Km = 9 × 10⁷ M⁻¹ min⁻¹ described above. The value of kcat/Km was determined in the presence of G, and that for koffS 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 koffS ≈ 10⁸ M⁻¹ min⁻¹ is significantly less than the rate of diffusional encounter of ~10¹¹ 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 pG2CCCUCU, 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 AG replacing the AS portion of G2CCCUCUAGp (T. McConnell and D. Herschlag, unpublished results). The 3' product of the reaction with the A3 substrate was not identified. The 3' product from the site-specific hydrolysis of the related substrate, G2CCCUCUAGUp*Cp, was observed to migrate faster than the product from the reaction with G, GAGUp*Cp; com-
comparison 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: $k_{\text{cat}}/K_m = 6 \times 10^7$ M$^{-1}$ min$^{-1}$; $K_m = 0.05$ min$^{-1}$; and $k_{\text{single turnover}} = k_{\text{G}} = 0.7$ min$^{-1}$ (Table I; data not shown). The value of $k_{\text{cat}}$ 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 $k_{\text{cat}}/K_m$ 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 at position 414 (Tanner & Cech, 1987). In mechanism 2, the first step is analogous to nucleotidytransfer 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 oligo-nucleotide 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 mechanism 2 is a hydrolysis reaction, analogous to the intramolecular hydrolysis of sequences of the type GpN that has been observed for an O-eliminated to give cleavage at a site 15 nucleotides from the 5'-end that is homologous to the 5'-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 at position 414 (Tanner & Cech, 1987). In mechanism 2, the first step is analogous to nucleotidytransfer 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 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 molecules that contain a 3'-terminal G residue.

FIGURE 3: Determination of the rate of substrate dissociation from the extent and rate of trapping $S^*$ (pAGUp*CCCUCUA) as $P^*$ in pulse-chase experiments. (A) Ribozyme (50 nM) and $S^*$ were incubated for 0.25 min followed by a "chase" with (O) or without (G) 50 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).
Scheme IV

\[
\begin{align*}
E+S' & \rightarrow k_\text{cat}(-G) \rightarrow E+P^* \\
S' & \rightarrow k_\text{off} \rightarrow E+S
\end{align*}
\]

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 \( k_\text{off} \) can be determined from these data in two ways:

1. Equation 2, which was derived from Scheme IV, relates the fraction of ES that proceeds to form P* [i.e., Frac(trapped)] to the rate constants for hydrolysis, \( k_\text{cat}(-G) \), and dissociation of substrate, \( k_\text{off} \).

   \[
   \text{Frac(trapped)} = \frac{k_\text{cat}(-G)}{k_\text{cat}(-G) + k_\text{off}^S} \tag{2}
   \]

   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) was 0.66/0.85 = 0.78 and the independently measured \( k_\text{cat}(-G) = 0.7 \text{ min}^{-1} \) gives \( k_\text{off}^S = 0.2 \text{ min}^{-1} \) with use of eq 2.

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

   \[
   k_{\text{obs}d} = k_\text{cat}(-G) + k_\text{off}^S \tag{3}
   \]

   first-order plot of the data of Figure 3A, gives \( k_{\text{obs}d} = 0.9 \text{ min}^{-1} \). This value and \( k_\text{cat}(-G) = 0.7 \text{ min}^{-1} \) give \( k_\text{off}^S = 0.2 \text{ min}^{-1} \), which is the same as the value obtained above from the product yield.

Six similar experiments with 10-100 nM E gave \( k_\text{off}^S = 0.16-0.24 \text{ min}^{-1} \) from the product yields (eq 2) and \( k_\text{off}^S = 0.19-0.25 \text{ min}^{-1} \) from \( k_{\text{obs}d} \) (eq 3).

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

\[

k_\text{cat} / K_m = k_\text{on}^S k_\text{cat}(-G) / (k_\text{off}^S + k_\text{cat}(-G)) \tag{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 \mu M substrate with 0.5 mM GTP catalyzed by 7 nM E. The slopes of these lines are the same, within error, and give \( k = 0.09 \text{ min}^{-1} \) 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 \( k_\text{cat} \) (Table I). Rate constants of 0.05-0.10 \text{ min}^{-1} were obtained in several independent experiments (not shown).

The intercept of \( \sim 5 \text{ nM} \) 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 occurs after chemistry. The rate constant \( k = 0.14 \text{ min}^{-1} \) following the burst is consistent with the value of 0.1 \text{ 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 (\( \sim 80\% \)) 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 \mu M) the rate constants for disappearance of substrate in a single turnover are \( \sim 3 \) and \( > 4 \text{ min}^{-1} \), respectively, much greater than the value of \( k_\text{cat} = 0.1 \text{ min}^{-1} \). Thus \( k_\text{cat} \) represents a step that occurs after chemistry. The possibility that the small observed value of \( k_\text{cat} \) 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 S' Product Fragment: The results described above show that the rate-limiting step with saturating substrate (i.e., "\( k_\text{cat} \) conditions") occurs after the chemical conversion. This step could be dissociation of P, dissociation of GA\(_3\), or a conformational change of the ternary E-P-GA\(_3\).
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 \( k_{\text{off}} \). 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 pppG2CCCUCUA5, which is used in the “chase”. Nucleophilic attack by products (e.g., G2CCCUCUA5) after G residues of substrates that contain G at the 5'-end has been observed (eq 5; B. Flanagan, F. Murphy, and T. R. Cech, 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 \( k_{\text{off}} \) 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 \( \mu \)M 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 \( k_{\text{off}} \) can be obtained from the fraction of P* that goes on to form P*2 and from the rate constant, \( k_{\text{off}} \), 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 \( [P*2]_{t=\infty}/([P*]_{t=\infty}+[P*2]_{t=\infty}) = k_2[S]/(k_2[S] + k_{\text{off}}) \) \( \) (6)

\[ k_{\text{off}} = k_2[S] + k_{\text{off}} \] \( \) (7)

\[ k_{\text{off}} = 0.12 \text{ min}^{-1} \] (first-order plot not shown). Eliminating \( k_2[S] \) from eqs 6 and 7, and then solving for \( k_{\text{off}} \), gives \( k_{\text{off}} = 0.055 \text{ min}^{-1} \). A value of 0.062 min\(^{-1} \) is similarly obtained from Figure 5B. Values of \( k_{\text{off}} = 0.063 \) and 0.070 min\(^{-1} \) were obtained in analogous experiments with 1.6 \( \mu \)M 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 with the G binding site at 0.8–3.2 \( \mu \)M (not shown). Since the G site is not saturated, and \( k_{\text{off}} \) does not vary with the concentration of S, \( k_{\text{off}} \) is the rate constant for dissociation of P from the binary complex E-P.

The value of \( k_{\text{off}} \) = 0.06 min\(^{-1} \) is the same, within error, as the value of \( k_{\text{off}} = 0.05-0.1 \text{ min}^{-1} \) 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 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** \( \) dissociates before P from the Ternary E-P-GA Complex. **Pulse-chase experiments analogous to those described in the previous section were carried out in the presence of 2.5 \( \mu \)M G in order to generate E-P*-GA; the amount of P*2 (eq 5) formed during the chase (t2; Scheme III) with ~4 \( \mu \)M unlabeled substrate was compared to the amount formed from
E-P*, which was generated from reaction in the absence of G. Both E-P*G2A2 and E-P* reacted during the chase to give end points with 70-80% of the P* converted to P2*. (The low concentration of G and the 10-fold dilution upon addition of the "chase" with unlabeled S prevent significant inhibition or reversal of P2* 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 P2* presumably involves binding of unlabeled S in the G binding site so that the formation of P2* strongly suggests that GAS dissociates before P. This does not provide evidence for a compulsory order of dissociation, only for a faster dissociation of GA2 than of P. There was no detectable lag in the conversion of S to P.

The reactions were followed for 260 min and were first order. The time expected to establish equilibrium between bound and free G is fast. A lower limit is koffG/G ∼ 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,C,CCUCA) was determined from inhibition by 40-120 nM P of the reaction of 20-25 nM E, ∼ 1 nM pppG,C,CCUCA, 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 = koffG/G [P] + koffG ≠ 10⁶ M⁻¹ min⁻¹ (40 nM) + 0.06 ± 4 min⁻¹; this gives t₁/₂ ∼ 0.2 min. Because the ribozyme concentration is well below saturation for the "mismatched" substrate, the inhibition is expected to give the value Kd(E-P) = K = 1 (±0.5) nM directly (see Scheme III of the following paper [Herschlag & Cech, 1990b]). This value and koffG/G = 0.06-0.1 min⁻¹ give koffG/G = 10 × 10⁶ M⁻¹ min⁻¹ [range (4-20 × 10⁶ M⁻¹ min⁻¹)]. This is the same, within uncertainty, as the value koffS/G = (9 ± 3) × 10⁷ M⁻¹ min⁻¹.

Michaelis Constant for the Substrate G,C,CCUCA2. The value of K = 1 nM for S is obtained from division of kcat = 0.1 min⁻¹ by kcat/Km = 9 × 10⁻⁷ M⁻¹ min⁻¹. Since kcat/G = koffG/G and kcat/Km = kcat/S, K equals koffG/G. Thus, K is not equal to the dissociation constant for E-S. Nevertheless, the value of K is fortuitously similar to Kd(E-G) = 2 nM because of the similar binding of S and P.

Evidence for Independent Binding of G,C,CCUCA2 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 in 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,C,CCUCA2 and G,C,CCGCUA2 in the presence and absence of G are consistent with the absence of an effect of G on the value of koffS/G (Herschlag and Cech, unpublished results). (On the basis of estimates of the errors in rate and equilibrium 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 GAS 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 kcatS/G with and without G (see above) suggests that G does not provide such a kinetic block. In addition, the value of kcatG/Km is the same with 100-2300 μM G (data not shown), concentrations both above and below the Kd(E-G) ∼ 0.5 mM (Herschlag & Cech, 1990b). Thus, the rate of substrate binding (kcatG/Km; 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 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 = 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) × 10³ 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 10⁵-10¹ 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, 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 Kd ∼ 0.5 mM (Herschlag & Cech, 1990b): k = (7 × 10⁴ M⁻¹ min⁻¹) (0.5 mM) = 350 min⁻¹. [The limits of uncertainty for Kd(E-G) of 0.3-1.1 mM give a range of values of k = 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 diffusion limit. If the binding of G were rate-limiting, then the rate constant for conversion of the ternary complex, k, would be
greater than \( \sim 350 \text{ min}^{-1} \). This is because \( k_e \) would need to be faster than \( k_{\text{off}} \) in order not to be rate-limiting, and a value of \( k_{\text{off}} = 350 \text{ min}^{-1} \) is obtained by assuming that the binding of \( G \) is rate-limiting with \( k_{\text{on}} = 7 \times 10^5 \text{ M}^{-1} \text{ min}^{-1} \) and \( K_d(G) = 0.5 \text{ mM} \) (Herschlag and Cech, following paper in this issue).

There is an alternative mechanism to that with \( k_e \) 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, \( E-P-GA_3 \), followed by rate-limiting dissociation of \( GA_3 \) from the ternary complex would give \( k_e \) that represents the equilibrium between the ternary complexes and the rate constant for \( GA_3 \) dissociation according to \( \text{eq 8} \). However, the following analysis shows that this mechanism is unlikely, as it would require binding of \( GA_3 \) to \( E-P \) to occur at \( \sim 10^9 \text{ M}^{-1} \text{ min}^{-1} \), which is \( \sim 10^6 \)-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 \( K_e \) for the equilibrium

\[
E-S + G = E-P + GA_3 \sim 1
\]

The equilibrium

\[
K_e = [E-P][GA_3]/[E-S][G]
\]

(9)

and

\[
K_3 = K_2K_G/K_dG
\]

(10)

and

\[
k_e = k_{\text{off}}GAS/K_3
\]

(11)

represented by \( K_3 \) is related to the equilibrium for the ternary complexes (\( K_5 \); \text{eq 8}) by the dissociation constants for \( G \) and \( GA_3 \) according to \text{eq 10}. Combining \text{eqs 10} and \text{8}, with use of \( k_{\text{off}}GAS = k_{\text{off}}G/k_{\text{on}}GAS \), gives \text{eq 11}. Solving \text{eq 11} for \( k_{\text{on}}GAS \) with use of \( k_e = 350 \text{ min}^{-1} \), \( K_e = 1 \), and \( K_3 = 0.5 \text{ mM} \) gives \( k_{\text{on}}GAS = 7 \times 10^3 \text{ M}^{-1} \text{ min}^{-1} \). The value of \( K_3 \approx 0.5 \text{ mM} \), which was determined for dissociation from the binary E-G complex (Herschlag and Cech, following paper), is used in \text{eq 11} for dissociation from the ternary complex because of the evidence that binding of \( S \) and \( G \) are independent (see above).

Thus, we adopt a model in which \( k_e \) represents a step or steps associated with the chemical conversion of the ternary complex, rather than the binding of \( G \) or the dissociation of \( GA_3 \).

**Discussion**

The ribozyme from \( T. \text{ thermophila} \) pre-tRNA catalyzes the sequence-specific RNA endonuclease reaction shown in \text{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 \( \sim 10^4 \)-fold stronger than expected for simple helix formation with the 5'-exon binding site (Figure 1B; Herlich and Cech, following paper). The binding of \( G \) with \( K_d \approx 0.5 \text{ mM} \) was determined from data in the following paper (Herschlag & Cech, 1990b).
- After formation of the ternary E-G complex, reaction occurs rapidly with \( k_e \approx 350 \text{ min}^{-1} \). 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 \( k_e \approx 350 \text{ min}^{-1} \) rather than dissociates \( k_{\text{off}} \).

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 \( 1 \text{ nM} \), respectively, show that the \( PA_3 \) 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 ScaI ribozyme used herein.) The absence of stabilization from the \( PA_3 \) 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 \( A \) moiety.

The dissociation constants of 1 and 2 nM for the oligonucleotide product and substrate, respectively, reflect \( \sim 10^4 \)-fold (\( \sim 6 \text{ kcal/mol} \)) stronger binding than calculated for base pairing between the oligonucleotide and GGAGG, 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 \( K_d \) equals \( K_e \) (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 \( 10^{-7} - 10^{-8} \text{ M}^{-1} \text{ min}^{-1} \) (Scheme I and Herschlag and Cech, following paper) are far below the diffusion limit of \( \sim 10^{11} \text{ M}^{-1} \text{ min}^{-1} \) for collision of small molecules (Eigen & Hammes, 1963). However, these observed binding rates are similar to the observed rate constants of \( \sim 10^{-7} - 10^{-8} \text{ M}^{-1} \text{ min}^{-1} \) for helix formation between two oligonucleotides (Porscherk & Eigen, 1971; Craig et al., 1971; Porschke et al., 1973; Raveches et al., 1974; Bruslauer & 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 S'-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 was 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 $k_{cat}/K_m \approx 10^7 M^{-1} min^{-1}$ (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 NH₄Cl, 0.1 M MgCl₂), 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 diffusion 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 aminocyl-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 $K_m$ 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 $10^7 M^{-1} min^{-1}$, 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 carboxylations 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 on 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 $k_c \approx 350$ min⁻¹ (Scheme I). However, if the binding of G were
slower than diffusion controlled by more than 10⁴-fold, so this binding was rate-limiting for \( k_{cat}/K_m \)⁰ (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 \( k_c = 350 \text{ min}^{-1} \) 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 Mg²⁺ 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 Mg²⁺ 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 phosphoreryl oxygen atoms at the cleavage site by sulfur (McSwiggen and 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, \( G_2CCCUCUA \), because binding and chemistry are each partially rate-limiting (Herschlag and Cech, following paper), studies of phosphate diesters in non-enzymatic 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 \( k_c \approx 350 \text{ min}^{-1} \) 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 \( k_{\text{uncat}} = 3 \times 10^{-9} \text{ min}^{-1} \) was estimated as follows: The rate constant for reaction of water with dimethyl phosphate monooanion was estimated from the rate constant for the reaction of hydroxide ion, \( k_{\text{OH}^-} = 8 \times 10^{-9} \text{ M}^{-1} \text{ min}^{-1} \) (attack at phosphate; 50°C; Kumamoto et al., 1956; Haake & Westheimer, 1961), the linear free energy relationship \( \beta_{\text{water}} = 0.3 \) (Kirby & Younas, 1970), and the concentration of water of 55 M with use of eq 12.

\[
\begin{align*}
\text{rate of} \ k_{\text{uncat}} &= 3 \times k_w = (55 \text{ M})(k_{\text{OH}^-}) 10^{-4}(\delta_{\text{water}}) (k_{\text{KOH}} - k_{\text{KROH}}) \\
\text{rate of} \ k_{\text{cat}} &= 10^{-4}(\delta_{\text{water}}) (k_{\text{KOH}} - k_{\text{KROH}})
\end{align*}
\]

The calculated rate constant of \( k_c \approx 350 \text{ min}^{-1} \) was then adjusted according to eq 13 with use of the linear free energy relationship \( \beta_{\text{water}} = 0.3 \) (Kirby & Younas, 1970) to account for the \( k_p \) of the ribosyl anion leaving group being \( \sim 3 \) lower than that of methoxide ion, the leaving group in the hydrolysis of dimethyl phosphate. It should be noted that \( k_{\text{uncat}} \) 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.

**What Limits the Rate of Self-Splicing?** The large value of \( k_c \approx 350 \text{ min}^{-1} \) at 50°C compared with the maximal rate of self-splicing of \( \sim 1 \text{ min}^{-1} \) 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 \( \sim 500\)-fold slower than the reaction of the ternary complex with guanosine bound \( [k_c/k_c(-G) = (350 \text{ min}^{-1})/(0.7 \text{ min}^{-1}) = 500] \). This suggests that interactions of the ribozyme with the guanosine away from the nucleophilic 3'-hydroxyl group account for \( \sim 4 \text{ kcal/mol} \) 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 \( k_c/k_{\text{uncat}} \approx 10^{11} \)-fold described above and the equation \( \Delta G^0 = \Delta G^0 \text{(in kJ/mol}) = (k_c/k_{\text{uncat}}) \text{ at 30°C} \). 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 \( k_{\text{cat}} = 0.1 \text{ min}^{-1} \) (Table I) is similar to the previously reported value of \( k_{\text{cat}} = 0.04 \text{ min}^{-1} \) (Zaug et al., 1988). However, the value of \( k_m = 1 \text{ nM} \) obtained herein is much different from the previous value of \( k_m = 160 \text{ nM} \). It is likely that folding of the ribozyme affected the earlier steady-state measurements, because the reactions were initiated by the addition of MgCl₂ to a denatured ribozyme in EDTA. Preliminary experiments have suggested that preincubation with Mg²⁺ does indeed cause a slow conformational change of the ribozyme to a more active form, so ribozyme was preincubated with Mg²⁺ 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, \( G_2CCCUCUA \), gave values of \( k_{\text{cat}} = 0.13 \text{ min}^{-1} \) and \( k_m = 70 \text{ nM} \) (McSwiggen & Cech, 1989), which are similar to and much lower than the respective values for \( G_2CCCUCUA \) 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 \( \sim 2 \)-fold. Thus, the value of \( k_{\text{cat}} \) should be rather accurate, but the value of \( k_m \) is not expected to be accurate. The values of \( k_{\text{cat}}/K_m \) and \( K_m(E-S) \) are the same for the \( G_2CCCUCUA \) and \( G_2CCCUCUA \) 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 $k_{\text{cat}} = 6 \text{ min}^{-1}$ and $K_m = 0.3 \mu M$ obtained for the “mismatched” substrate, $G_3C_5C_3C_2C_1G_4$, in the following paper (Herschlag & Cech, 1990b) are similar to the previously reported values of $k_{\text{cat}} = 1-3 \text{ min}^{-1}$ and $K_m = 0.3-0.7 \mu M$ for this and the related substrate, $G_3C_3C_5C_1G_4$ (Zaug et al., 1988; McSwiggen & Cech, 1989). The weaker binding of the mismatched substrate ($K_d = 2.5 \mu M$) 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 Scale 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 ($K_d = 2 \text{ nM}$) 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 of the ribozyme. We suggest a physical picture in which the “mismatched” substrate, G$_2$CCCGCUA$_5$, in the following paper (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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