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Research Articles

Stereochemistry of RNA Cleavage by the *Tetrahymena* Ribozyme and Evidence That the Chemical Step Is Not Rate-Limiting

JAMES A. MCSWIGGEN AND THOMAS R. CECH

The intervening sequence of the ribosomal RNA precursor of *Tetrahymena* is a catalytic RNA molecule, or ribozyme. Acting as a sequence-specific endoribonuclease, it cleaves single-stranded RNA substrates with concomitant addition of guanosine. The chemistry of the reaction has now been studied by introduction of a single phosphorothioate in the substrate RNA at the cleavage site. Kinetic studies show no significant effect of this substitution on k_{cat} (rate constant) or K_m (Michaelis constant), providing evidence that some step other than the chemical step is rate-limiting. Product analysis reveals that the reaction proceeds with inversion of configuration at phosphorus, consistent with an in-line, S_N2 (P) mechanism. Thus, the ribozyme reaction is in the same mechanistic category as the individual displacement reactions catalyzed by protein nucleotidyltransferases, phosphotransferases, and nucleases.

THE NUCLEAR PRECURSOR TO RIBOSOMAL RNA IN *Tetrahymena thermophila*, a ciliated protozoan, contains a 413-nucleotide intervening sequence (IVS). The IVS excises itself from the larger RNA in a protein-independent reaction called self-splicing (*1*).

Self-splicing occurs through two transesterification reactions (exchanges of phosphate esters which leave the total number of phosphodiester bonds unchanged). In the first transesterification, the 5′ splice site is cleaved as guanosine is added to the 5′ end of the IVS (*2*); guanosine, GMP, and GTP (guanosine mono- and triphosphate, respectively) have similar activities as substrates. It has been proposed that the guanosine acts as a nucleophile in an in-line, S_N2 (P) reaction (*3, 4*), but there has been no direct test of this mechanism. In the second transesterification step, the 3′ splice site is cleaved as the exons (RNA sequences flanking the IVS) are joined.

The excised IVS RNA retains catalytic activity (*5, 6*). Truncated versions of the IVS RNA act as RNA enzymes (ribozymes) to cleave, join, or dephosphorylate RNA substrates (*7–9*). The sequence-specific endoribonuclease activity of the *Tetrahymena* ribozyme (Fig. 1) is an intermolecular version of the first step of preribosomal RNA self-splicing. The site of substrate cleavage is determined by a base-pairing interaction; the substrate binds to the same sequence within the IVS that specifies the 5′ splice site during self-splicing (*9–11*). The endoribonuclease reaction facilitates detailed studies of the chemistry of guanosine addition because the substrate can be provided as an oligonucleotide. Oligonucleotides are easily synthesized with a variety of sequences and functional group substitutions.

Recently we studied cleavage of substrates that had single-base changes several bases preceding the cleavage site, giving mismatched substrate-ribozyme complexes. Surprisingly, mismatches greatly enhanced the rate of cleavage (*12, 13*). One reasonable explanation was that the mismatches might be facilitating a rate-limiting conformational change (*14*) rather than affecting the chemical step. Such a model gives a strong prediction; an alteration of the phosphate at the cleavage site that greatly decreases its reactivity toward O-P bond cleavage might have very little effect on the rate of the reaction. This prediction has now been tested by substitution of a phosphorothioate at the reaction site. The finding of very little change in cleavage rate, even with the best substrates, supports the model of a non–rate-limiting chemical step.

The phosphorothioate-containing substrates also provide a test for the stereochemical course of the reaction. We find that the reaction proceeds with inversion of configuration at phosphorus, the same result obtained for most proteins that catalyze transesterification of phosphate esters (*15, 16*). This stereochemistry suggests

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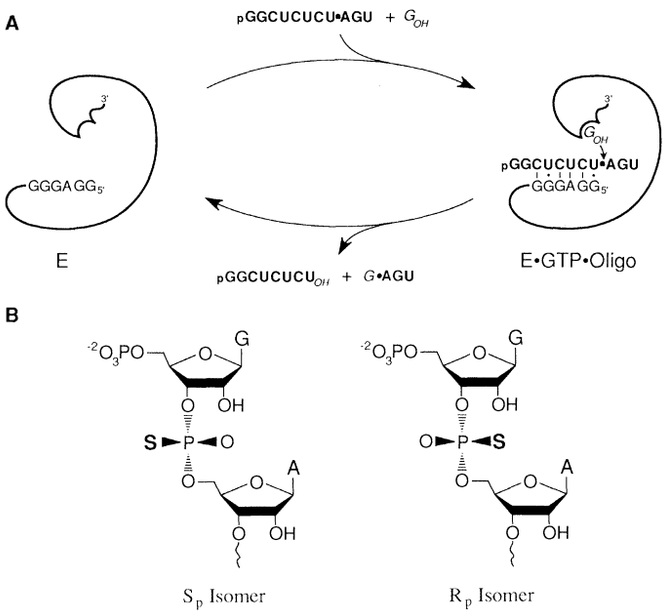

Fig. 1. Model for the endoribonuclease activity of the *Tetrahymena* ribozyme on normal and phosphorothioate-containing substrates. (**A**) The ribozyme (E) is the L−21 Sca I RNA, a shortened version of the *Tetrahymena* IVS RNA missing 21 nucleotides from its 5′ end and 5 nucleotides from its 3′ end (*12*). The ribozyme binds two substrates, a guanosine (or GTP; italic *G*) and an oligoribonucleotide (bold letters; dot represents cleavage site phosphate or phosphorothioate). Cleavage occurs by a transesterification reaction in which the G becomes attached to the 5′ end of the 3′ cleavage fragment. The ribozyme is then recycled. (**B**) Configuration of the two isomers of the phosphorothioate diester, the R_p isomer being that produced by RNA polymerase during the transcription reaction.

that the reaction does not involve a covalent substrate-enzyme intermediate. It is consistent with nucleophilic attack by the 3′-hydroxyl of guanosine in an in-line, S_N2 (P) reaction as proposed earlier (*3, 4*).

A phosphorothioate linkage at the cleavage site does not substantially alter the reaction rate. Two criteria were used in designing the oligonucleotide substrates shown in Table 1. (i) The substrates should contain only a single adenosine residue, located at the 3′ side of the cleavage site, so that a single phosphorothioate linkage could be introduced by replacing adenosine triphosphate (ATP) with *S*p-ATPαS in the transcription mixture. Second, the substrates should span the range of cleavage rates observed earlier (*12*) so that the effect of phosphorothioate substitution at the cleavage site could be examined over the entire range.

The time course of the endoribonuclease reaction with 5′ end-labeled pGGCCCGCU(P)AGU and pGGCCCGCU(S)AGU as substrates (*17*) was examined (Fig. 2A). Both substrates are cut readily to yield a single labeled product (pGGCCCGCU). Control experiments show that more than 95 percent of the phosphorothioate-containing substrate can be converted to product (*18*) and that ~97 percent of this substrate contains a phosphorothioate linkage at the cleavage site (see below). Thus the L-21 Sca I ribozyme can readily cleave the phosphorothioate linkage.

From an analysis of the cleavage rate as a function of the substrate concentration for the phosphate-containing and phosphorothioate-containing substrates (Fig. 2B), it is clear that V_{max} (1/slope on this plot) for the phosphorothioate substrate is slower than for the substrate containing phosphate at the cleavage site, but the effect is small (see Table 1). In addition, K_m is slightly reduced by the presence of the phosphorothioate linkage. The net effect is that the ratio k_{cat}/K_m, which is the apparent second-order rate constant for reaction of free enzyme and free substrate (*19*), remains essentially unchanged by the introduction of the phosphorothioate. A similar conclusion is drawn for the three other substrate pairs that have been examined (Table 1). In each case, k_{cat} is reduced by the introduction of phosphorothioate, but only by 40 to 50 percent; at the same time k_{cat}/K_m remains virtually unchanged.

Inversion of configuration at phosphorus. The 3′ fragments produced by endoribonuclease cleavage were examined to confirm that the phosphorothioate-linked product actually contained the phosphorothioate linkage and to determine the stereochemical course of the reaction. RNA polymerase utilizes only the S_p isomers of nucleoside triphosphates containing phosphorothioate at the alpha position; the phosphorothioate is converted to the R_p isomer upon incorporation into RNA (*20–22*). The ribozyme-catalyzed reaction could occur with retention of this R_p configuration or with inversion to the S_p configuration. To determine the configuration, the 3′ cleavage products were digested by ribonuclease (RNase) T_1 and snake venom phosphodiesterase (*23*). These enzymes cut the R_p phosphorothioate isomer almost exclusively (500 to 2000 times faster than the S_p isomer in the case of snake venom phosphodiesterase) (*21, 24, 25*).

The 3′ cleavage products (pG(P)AGU and pG(S)AGU) were obtained by reacting phosphate- and phosphorothioate-containing substrates with 5′-[^{32}P]GMP and isolating the products on 24 percent polyacrylamide gels. As a control, the same tetranucleotides were produced by transcription so that pG(S)AGU of known configuration (R_p) could be obtained (*26*). On a 24 percent polyacrylamide gel, the reaction product pG(S)AGU migrates slightly slower than either the transcribed pG(S)AGU or pG(P)AGU (Fig. 3, A and B), suggesting that the configuration about the phosphorothioate linkage has been altered in the product of ribozyme cleavage. During purification of the pG(S)AGU product, a minor band was seen with a gel mobility, sequence, and RNase digestion pattern consistent with it being a pG(P)AGU contaminant (*18*). The minor band constituted 6 percent of the total sample as determined by radioanalytic imaging. This suggests that roughly 3 percent of the phosphorothioate-linked substrate is actually phosphate-linked contaminant, since less than 10 percent of

Table 1. Comparison of rate constants for phosphate and phosphorothioate containing oligonucleotide substrates. Reactions were performed as in Fig. 2. The first three columns list measured k_{cat}, K_m, and k_{cat}/K_m for the phosphate-containing substrate. The ratios of these constants for the phosphate (P) and thiophosphate (S) substrates are listed in the remaining columns. Three independent determinations of the kinetic parameters for the fourth substrate revealed that the absolute values can vary by as much as a factor of 2 between determinations. The (S)/(P) ratios were less variable (±50 percent).

Oligonucleotide	k_{cat} (P) (min^{-1})	K_m (P) (μM)	k_{cat}/K_m (P) [$(min \cdot \mu M)^{-1}$]	$\frac{k_{cat}\ (S)}{k_{cat}\ (P)}$	$\frac{k_m\ (S)}{k_m\ (P)}$	$\frac{k_{cat}/K_m\ (S)}{k_{cat}/K_m\ (P)}$
pGGCCCUCU·AGU	0.13	0.07	2.0	0.63	0.48	1.3
p - - - U - - - - · - - -	0.27	0.10	2.7	0.60	0.44	1.4
p - - - - - C - - · - - -	2.0	0.30	6.5	0.47	0.66	0.7
p - - - - - G - - · - - -	3.0	0.66	4.5	0.48	0.64	0.75

oligonucleotide substrate has been converted to product in these preparations, and since phosphate containing oligonucleotide is cleaved at roughly twice the rate of phosphorothioate containing oligonucleotide under those conditions. Such a level is consistent with the stated purity of ATPαS used to make these oligonucleotides.

Hydrolysis of the pG(P)AGU and pG(S)AGU oligonucleotides by RNase T1 and snake venom phosphodiesterase is shown in Fig. 3. Digestion of pG(P)AGU was the same whether it was produced by ribozyme reaction or by transcription, and therefore only pG(P)AGU produced by ribozyme action is included here. In contrast, treatment of the pG(S)AGU ribozyme product is much different from that of the control pG(S)AGU transcription product by both nucleases. RNase T1 cuts after guanosines so that cleavage of pG(P)AGU yields two labeled products at early times, reducing to pGp at longer times (Fig. 3A). Snake venom phosphodiesterase cuts at every linkage to produce three product bands (Fig. 3B) which are ultimately digested to GMP (*18*). Transcribed pG(S)AGU is cut about twice as slowly as pG(P)AGU by RNase T1 and only slightly more slowly by phosphodiesterase, but yields the same products at longer times. In contrast, the pG(S)AGU produced by the ribozyme reaction yields only the trinucleotide, pG(S)AGp, when cut with RNase T1 and the dinucleotide, pG(S)A, when cut with phosphodiesterase. Thus, the ribozyme product pG(S)AGU

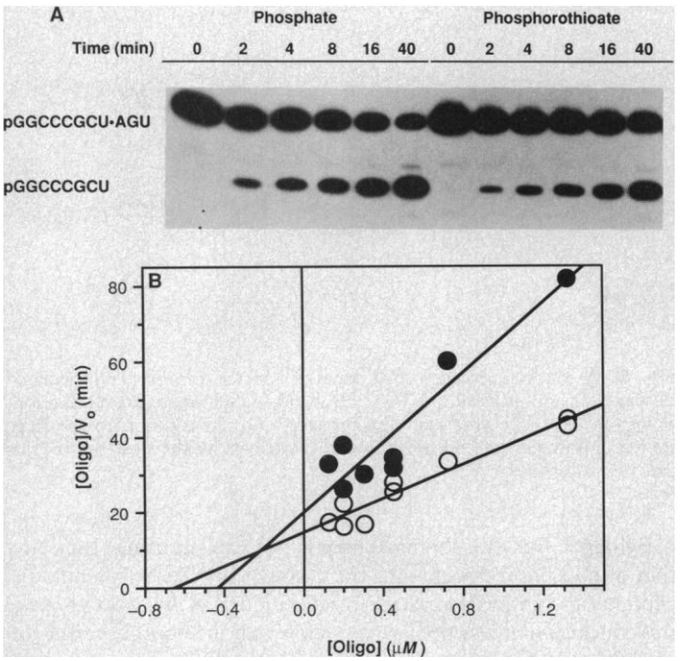

Fig. 2. Kinetic analysis of the endoribonuclease cleavage reaction on oligonucleotides containing a phosphate or phosphorothioate linkage at the cleavage site. (**A**) Sample kinetics time course with 1 μ*M* 5′ end-labeled pGGCCCGCU(P)AGU or pGGCCCGCU(S)AGU substrates along with 35 n*M* L−21 Sca I IVS and 500 μ*M* GTP in TENM buffer (50 m*M* tris, *p*H 7.5, 0.01 m*M* EDTA, 10 m*M* NaCl, 10 m*M* $MgCl_2$); incubations were at 50°C for the times indicated. The L−21 Sca I ribozyme and oligonucleotide substrates were prepared by T7 transcription as described (*12*); preparation of phosphorothioate containing substrates included 1 m*M* ATPαS (Amersham) in place of ATP in the transcription mixture, and 5 m*M* dithiothreitol in storage buffers. Reactions were carried out by heating separate solutions of 2× oligonucleotide solution and a 2× solution containing ribozyme plus GTP (both in TENM buffer) to 50°C for 5 minutes, mixing 15 μl of each, and incubating at 50°C. Reactions were stopped by removing 3 μl of reaction mixture into 3 μl of stop mixture [15 m*M* EDTA, 0.1× TBE (tris, borate, EDTA), 0.05 percent xylene cyanol, 95 percent formamide] and placing on dry ice. Samples were subjected to electrophoresis on 20 percent polyacrylamide, 8*M* urea gels. (**B**) Plot of the ratio of the oligonucleotide concentration to the initial velocity as a function of the oligonucleotide concentration for the substrates pGGCCCGCU(P)AGU (open circles) or pGGCCCGCU(S)AGU (filled circles). Slope = $1/V_{max}$; x intercept = $-K_m$. Reactions were performed as in part A except that the final reaction mixture contained 15 n*M* L−21 Sca I IVS and 1 m*M* GTP in TENM buffer, along with the indicated concentrations of oligo substrate. Gels were quantitated with the use of an AMBIS radioanalytic scanner. Initial velocities were determined from a linear least-squares fit through points representing up to the first 10 percent of the reaction.

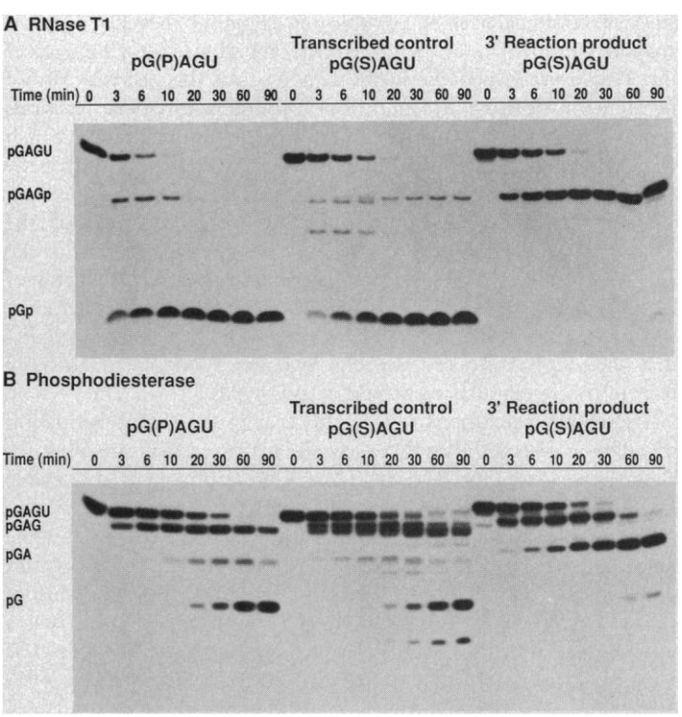

Fig. 3. Demonstration that the 3′ product of the cleavage reaction contains a phosphorothioate linkage that is stereochemically inverted relative to the substrate. (**A**) RNase T1 digestion of pG(P)AGU and pG(S)AGU produced by transcription and by ribozyme cleavage. The 5′ end-labeled oligonucleotide was mixed with RNase T1 (10^{-5} U/μl) (Pharmacia) in 50 m*M* tris (*p*H 7.5), 0.1 m*M* EDTA, and yeast transfer RNA at 0.25 mg/ml, then incubated at 37°C. Portions of the reaction were mixed with stop mixture and placed on dry ice at the indicated times. Samples were analyzed on 24 percent polyacrylamide, 8*M* urea, 1× TBE gels. Labeled pG(P)AGU and pG(S)AGU were prepared by incubation of 5′-[^{32}P]GMP (~1.3 μ*M*) with 100 nm of L−21 Sca I IVS and 6.8 μ*M* pGGCCCGCU(P)AGU or pGGCCCGCU(S)AGU in TENM buffer at 50°C for 2 hours. This resulted in approximately 8 percent of the initial oligonucleotide substrate being converted into labeled pG(P)AGU or pG(S)AGU. The tetranucleotide products were separated on a native gel (24 percent polyacrylamide, 1× TBE), excised, eluted into TE (tris-EDTA), and used without further purification. The control pG(S)AGU was prepared by transcription (*12, 25*) and 5′ end-labeled with [γ-^{32}P]ATP. (**B**) Snake venom phosphodiesterase digestion of pG(P)AGU and pG(S)AGU produced by transcription and by ribozyme cleavage. Digestions were as in (A) except that snake venom phosphodiesterase (5×10^{-6} U/μl) (Sigma) was used in place of RNase T1 and 10 m*M* $MgCl_2$ was included in the digestion mix.

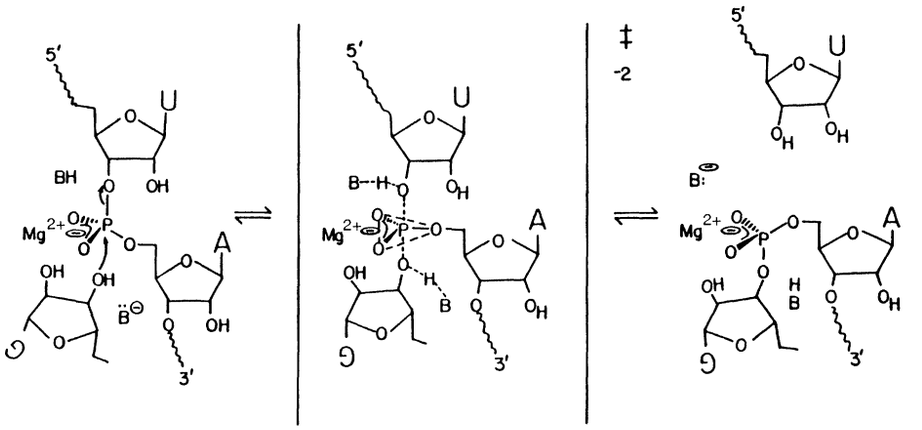

Fig. 4. Model for ribozyme-catalyzed cleavage of the UpA bond in the oligonucleotide substrate (*4*). The 3′-hydroxyl group of guanosine acts as the nucleophile in an S_N2 (P) reaction. Inversion of configuration around phosphorus is the stereochemical course demonstrated above. The general acid-base catalysis performed by BH and B− is hypothetical, as is the coordination of Mg^{2+} to the phosphate, stabilizing the transition state. ‡, trigonal bipyramid transition state with −2 net charge.

has the resistance to digestion indicative of the S_p isomer at the phosphorothioate linkage instead of the reactivity indicative of the original R_p isomer.

Implications of the stereochemical course of the reaction. We find that the endoribonuclease reaction of the *Tetrahymena* ribozyme proceeds with inversion of configuration at the phosphate. This conclusion is based on the knowledge that the substrate contained the R_p isomer of the phosphorothioate—since RNA polymerase generates transcripts containing only the R_p isomer—and that the product was resistant to digestion by two nucleases, RNase T1 and snake venom phosphodiesterase. These nucleases, while mechanistically quite distinct (*23*), share stereochemical specificity for the R_p isomer and have little or no activity on the S_p isomer. A direct stereochemical analysis has not been done on these products.

Inversion of configuration is best explained by a single nucleophilic substitution reaction (*16*). Nucleophilic attack by the 3′-hydroxyl of guanosine in an in-line, S_N2 (P) reaction (Fig. 4) has been proposed earlier for the mechanism of action of the *Tetrahymena* ribozyme (*3, 4*). The observation that the reaction proceeds with inversion of configuration at the phosphate is consistent with this model.

The fact that the endoribonuclease reaction proceeds with inversion of configuration at phosphorus suggests that no covalent enzyme intermediate exists in the reaction pathway (*16*). This places the *Tetrahymena* ribozyme in the same class as the vast majority of (protein) nucleotidyl and phosphoryl transferases whose reactions proceed with inversion of configuration at phosphorus and which do not form covalent enzyme intermediates. It is important that the endoribonuclease reaction discussed in this article not be confused with the polymerization and phosphotransfer reactions of the *Tetrahymena* ribozyme (*8, 9*); these reactions require a form of the ribozyme that can form a covalent intermediate between a portion of the substrate and the 3′ end of the ribozyme. The stereochemistry for these reactions has not been determined, but we would predict that they would proceed through two inversions so that overall retention of configuration would be observed.

The above conclusions rest on the assumption that introduction of sulfur in place of phosphate oxygen at the reaction site does not alter the reaction mechanism. This assumption seems reasonable since (i) it is unlikely that the active site of the ribozyme has two mechanistically distinct reaction pathways available to it; (ii) while minor reaction pathways might exist, it would be difficult to explain the similarity in rates between phosphate and phosphorothioate substrates if the phosphorothioate were cleaved exclusively by such a minor pathway; and (iii) all other enzymes that have been checked utilize the same reaction pathway regardless of whether a phosphoryl or a thiophosphoryl group is transferred (*15, 16*).

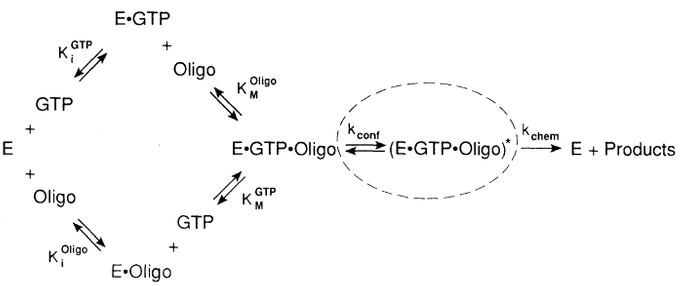

Fig. 5. A random, biomolecular kinetics scheme for the IVS-mediated cleavage of oligonucleotides by GTP, featuring a rate-limiting conformational step prior to the chemical cleavage step (*32*). In addition to the steps shown, a slow product release step may contribute to the slow turnover of matched substrates.

Evidence that the chemical step is not rate-limiting. Introduction of phosphorothioate into the cleavage site has only a minimal effect on the endoribonuclease reaction kinetics. In order to make this conclusion, it was necessary to show that the phosphorothioate-containing substrate had not lost its modified linkage during preparation (*27*). Each substrate was tested for the presence of phosphorothioate after its synthesis, but a more sensitive test was to examine the 3′ product for contaminating phosphate. At early times in the reaction, it was found that only 6 percent of the 3′ oligonucleotide product—thus only 3 percent of the original substrate—had a gel mobility and an RNase sensitivity consistent with it being contaminating phosphate-containing product.

On the basis of the reactivity of phosphate and phosphorothioate *triesters*, it has been suggested that introduction of phosphorothioate in a diester should slow the chemical step of a bimolecular nucleophilic displacement reaction by 30- to 100-fold (*28–30*). In the nucleophilic displacement reaction catalyzed by the *Tetrahymena* ribozyme, we observed only a twofold decrease in reaction rate. Our explanation is that the chemical step is not rate-limiting, thus a dramatic rate decrease in the chemical step does not greatly affect k_{cat}. Such a situation has been proposed for the myosin adenosine triphosphatase (*31*), as well as for the Klenow fragment of DNA polymerase I (*30, 32*).

In a different class of RNA-catalyzed reaction, the autolytic cleavage of the positive-strand RNA from satellite tobacco ringspot virus, introduction of phosphorothioates at multiple sites including the cleavage site greatly reduced the rate of cleavage (*33*). Studying a similar reaction with an RNA that has a single phosphorothioate substitution, Dahm and Uhlenbeck (*34*) have measured a 180-fold reduction in cleavage rate, suggesting that the chemical step is rate-limiting in this case. In contrast, the rate of autolytic cleavage and

ligation of the negative strand of the satellite RNA is only slightly affected by phosphorothioate substitution at the reaction site (*35*). Presumably here, as with the *Tetrahymena* ribozyme, the chemical step is not rate-limiting.

What might be rate-limiting if not the chemical step? In the case of the *Tetrahymena* ribozyme, Zaug and co-workers (*12*) have proposed a rate-limiting conformational change in the ribozyme-substrate interaction. This proposal was based on the observation that oligonucleotide substrates that form mismatched base-pairing with the binding site internal to the IVS (5′-GGAGGG in Fig. 1) exhibit a much higher cleavage rate than substrates that are completely paired with the internal binding site. The oligonucleotide substrate may have to be partially melted from the internal binding site prior to entering the transition state for the chemical step (*14*). Our working model for the endoribonuclease cleavage reaction is shown in Fig. 5. We propose that binding of oligonucleotide and guanosine occurs by a random, rapid equilibrium reaction, and that a rate-limiting conformational step precedes the chemical step (*36*).

The conclusion that the chemical step is not rate-limiting has a major impact on the interpretation of previous kinetic data. For example, we have reported that inosine and 2-aminopurine ribonucleoside have the same k_{cat} as guanosine for the first step of self-splicing (*3*). This equivalence may simply mean that neither of these substitutions affects a rate-limiting conformational change (*37*). Furthermore, site-specific mutations in the ribozyme often decrease k_{cat}. We must now consider that these base substitutions could be slowing a conformational step rather than interfering with the chemical step.

Other reactions carried out by the *Tetrahymena* ribozyme are also relatively slow, most with rates ≤ 1 min^{-1} (*38, 39*). Thus, it is entirely possible that all of these rates reflect conformational changes in the ribozyme or the substrate-ribozyme interaction, rather than intrinsic rates of nucleotidyltransfer or phosphotransfer catalyzed by the *Tetrahymena* ribozyme.

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27. RNA and DNA phosphorothioate linkages are readily alkylated, making them more labile to hydrolysis [B. A. Connolly and F. Eckstein, *Biochemistry* **18**, 592 (1982); F. H. Westheimer, *Science* **235**, 1173 (1987)]. However, the main reaction occurring to alkylated phosphorothioate linkages seems to be a loss of sulfur and conversion to the natural, phosphate-linked nucleic acid [G. Gish and F. Eckstein, *Science* **240**, 1520 (1988)]. Thus our concern was primarily with the possibility that the phosphorothioate containing oligonucleotides had been converted back to the phosphate-linked nucleic acid.
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