Evidence for the Rate-Limiting Departure of the 5′-Oxygen in Nonenzymatic and Hammerhead Ribozyme-Catalyzed Reactions

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Abstract: Kinetic analysis, using substrates that consisted entirely of deoxynucleotides with the exception of the single mandatory ribonucleotide at the cleavage site which contained either a 5′-oxy- or 5′-thio-leaving group, demonstrated that the departure of the 5′-leaving group was not the rate-limiting step of a hammerhead ribozyme-catalyzed reaction [Kuimelis, R. G.; McLaughlin, L. W. *J. Am. Chem. Soc.* **1995**, *117*, 11019−11020]. We recently synthesized a natural *all*-RNA substrate that contains a 5′-thio-leaving group at the cleavage site and performed detailed kinetic analysis. In contrast to the conclusion of Kuimelis and McLaughlin, we found that (i) the attack by the 2′-oxygen at C_{17} on the phosphorus atom is the rate-limiting step only for the substrate that contains a 5′-thio group (R11S) and (ii) the departure of the 5′-leaving group is the rate-limiting step for the natural *all*-RNA substrate (R11O) in both enzymatic and nonenzymatic reactions.

Introduction

Among various catalytic RNAs, the hammerhead-type ribozyme is the smallest and best-understood as far as the relationship between structure and function is concerned.[1−5] Over the past few years, it has become apparent that ribozymes are metalloenzymes.[6−15] Although recent X-ray analysis of a hammerhead ribozyme identified one potential catalytic metal ion, the exact mechanism of catalysis remains obscure.[16,17] Kuimelis and McLaughlin recently synthesized a DNA substrate that contained a single mandatory ribonucleotide with a 5′-thio-leaving group at the cleavage site for the hammerhead ribozyme.[18] Since (i) their 5′-thio substrate was cleaved at a rate similar to that of cleavage of the parental 5′-oxy substrate and (ii) there was no switch in metal ion specificity, they concluded that (i′) the departure of the 5′-leaving group is not the rate-limiting step of the hammerhead ribozyme-catalyzed reaction and (ii′), in contrast to the reaction catalyzed by the *Tetrahymena* ribozyme,[7] a metal cofactor does not interact with the leaving group in the transition state.

We have been working independently on a similar substrate with a 5′-thio-leaving group at the cleavage site. The major difference is that our substrate consists entirely of RNA whereas that of Kuimelis and McLaughlin consisted primarily of DNA. Hammerhead ribozymes can cleave natural RNA substrates more efficiently than their DNA counterparts. We will demonstrate in this paper that our kinetic parameters, based on reactions with an RNA substrate (see Figure 1 for the structures) that possesses either the native (R11O) or thio (R11S) linkage, are quite different from those based on a DNA substrate. Since we used natural *all*-RNA substrates and since our R11S was hydrolyzed by the ribozyme more than three orders of magnitude more rapidly than the corresponding DNA substrate of Kuimelis and McLaughlin (upon correction of pH and metal concentration to the same value), we believe that our result reflects true ribozyme-mediated catalysis.

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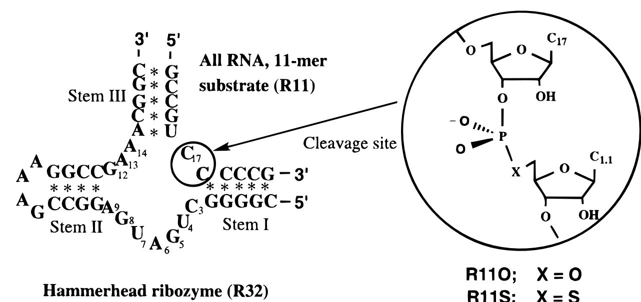

Figure 1. Sequence and secondary structure of a complex between 32-mer ribozyme (R32) and its *all*-RNA 11-mer substrate (R11O or R11S). An expanded view of the cleavage site (between C_{17} and $C_{1.1}$) is shown to provide details of the phosphodiester linkage in the unmodified substrate (R11O) and in the modified (R11S) substrate with a phosphorothioate linkage.

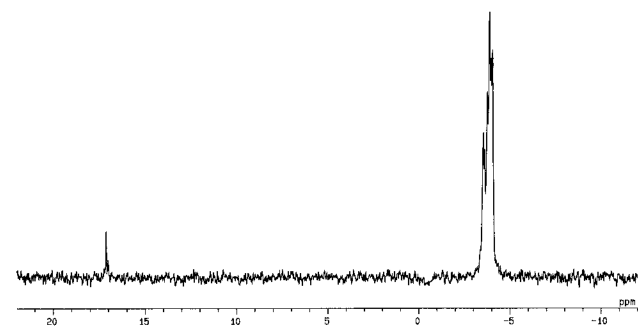

Figure 2. ^{31}P NMR spectrum of R11S. NMR experiments were performed with a model ALPHA-500 spectrometer as described previously (JEOL, Tokyo; 200 MHz for ^{31}P).[22] The ^{31}P chemical shift for the bridging thiolinkage was determined to be 17.12 ppm. The ^{31}P NMR spectrum was collected in H_2O-D_2O (4:1, v/v) at 5 °C.

Materials and Methods

Synthesis of Ribozymes and an Unmodified RNA Substrate. Ribozymes (R32, R31, and I-R32) and an unmodified RNA substrate (R11O) were synthesized with a DNA/RNA synthesizer (model 392; Applied Biosystems, Foster City, CA) and purified on a 20% polyacrylamide gel as described elsewhere.[19,20] RNA reagents were purchased from American Bionetics Inc. (ABN; Hayward, CA). All other reagents were purchased either from ABI or ABN. Purification of the synthesized oligonucleotides was performed as described in the User Bulletin from ABI (No. 53; 1989) with minor modifications.

Synthesis of the 5′-Phosphorothioate RNA Substrate. A bridging 5′-phosphorothioate linkage in a substrate (R11S) was incorporated at a specific site (between C_{17} and $C_{1.1}$; Figure 1) using 5′-thiol amidite. The coupling time was 600 s for 5′-thio rC and the following ribose amidite was coupled for 800 s. The sample was then base-deprotected as usual and treated with TEA/HF solution at 65 °C for 0.5 h instead of the standard 1.5 h.[21] The product was isolated from the desilylation reagent by precipitation with 3 M sodium acetate and butanol. The resulting 5′-thio substrate (R11S) was purified on a 20% polyacrylamide gel without labeling with ^{32}P. R11S was characterized by a partial sequencing and also by a ^{31}P NMR spectrum[22] that gave a well resolved signal at 17.12 ppm for the bridging 5′-phosphorothioate (Figure 2).

Cleavage of R11O and R11S Substrates by Ribozymes. For 5′-end-labeling of the R11S substrate, a small aliquot of the synthetic R11S substrate was incubated in buffer [50 mM Tris−HCl (pH 6.0), 10 mM $MgCl_2$, 0.1 mM EDTA (pH 8.0)] with polynucleotide kinase (*E. coli* A 19; Takara Biomedicals, Kyoto, Japan) and γ-^{32}P ATP (10 μCi/μL, Amersham, UK) for 30 min at 37 °C. After completion of the incubation, the reaction products were extracted twice with phenol and chloroform (1:1) and precipitated in ethanol in the presence of sodium acetate. We adopted this rapid purification strategy to avoid degradation of R11S substrate during electrophoretic separation and isolation. The labeled R11S substrate was stored dry at −80 °C until further use. The R11O substrate was 5′-end-labeled as reported elsewhere.[19]

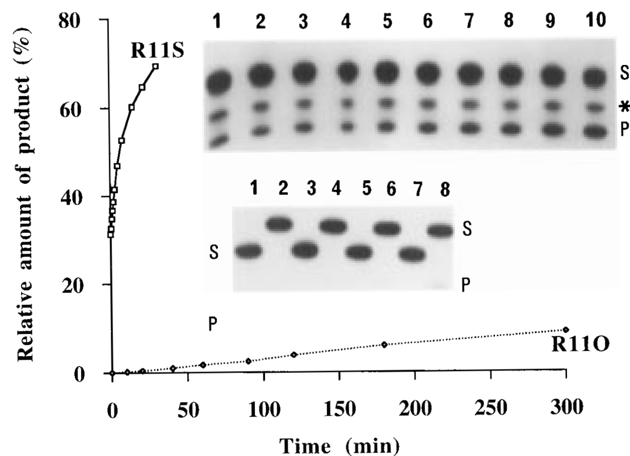

Figure 3. Time course of the R32 ribozyme-catalyzed reaction with either R11O or R11S as substrate in the presence of Mg^{2+} ions. The upper autoradiogram represents the ribozyme-mediated cleavage of R11S (lanes 1−10 for 0, 0.5, 1.0, 1.5, 2.0, 3.0, 5.0, 8.0, 15.0 and 22.0 min, respectively) and the lower one represents that of R11O (lanes 1−8 for 10, 20, 40, 60, 90, 120, 180, and 300 min, respectively). The R32 ribozyme-catalyzed cleavage reaction was carried out by incubating R11O (25 μM) and R32 (2.0 μM) in 50 mM MES buffer (pH 6.0) with 0.3 mM Mg^{2+} ions at 28 °C. In the lower autoradiogram, samples of lanes with odd numbers were loaded first and electrophoresis was started. Then samples of lanes with even numbers were added and electrophoresis was re-started. The extent of cleavage was determined by quantitation of radioactivity in the bands of substrate and product with a Bio-Image Analyzer (BA2000; Fuji Film, Tokyo).

Kinetic Measurements. Cleavage reactions were carried out in 50 mM MES buffer (pH 6.0) in the presence of 0.3 mM Mg^{2+} ions at 28 °C under multiple-turnover conditions. Concentrations of ribozyme and substrate were 2.0 μM and from 2 to 50 μM, respectively. The reaction was initiated by the addition of Mg^{2+} ions and aliquots were removed from the reaction mixture at appropriate intervals. These aliquots were then mixed with an equal volume of a solution that contained 100 mM EDTA, 9 M urea, and xylene cyanol and bromophenol blue (0.1% each). Uncleaved substrate and 5′-cleaved products were separated on a 20% polyacrylamide gel that contained 7 M urea (Figure 3). Electrophoresis was carried out with circulating iced water on the backside of the gel to prevent the decomposition of substrates. The extent of cleavage reactions was determined by quantitation of radioactivity in the bands of substrate and product with a Bio-image analyzer (BAS 2000; Fuji Film, Tokyo). Cleavage rates were obtained from the slopes of the curves for the time-course of reactions at the initial stage (0−3 min), and K_m and k_{cat} were calculated from Eadie-Hofstee plots (Figure 4).

All kinetic measurements were carried out under multiple-turnover conditions. For measurements of initial rates, for subsequent calculations of k_{cat} and K_m, at different concentrations of substrate (4−6 different concentrations, spanning the K_m), the first 3 min of the reaction, with six experimental points, were examined. Initial rates were measured in triplicate at 5−6 different concentrations of substrate and the average values were plotted (Eadie−Hofstee plots in Figure 4). Calculated values of k_{cat} and K_m are summarized in Table 1. Potential errors in these values were found to be 30% at most from results of duplicate experiments (two sets of Eadie−Hofstee plots).

During the isolation/phosphorylation of R11S, cleavage occurred not only at the expected labile P−S bond, to yield 6-mer and 5-mer products (cleavage after GUC), but also at the second phosphodiester linkage

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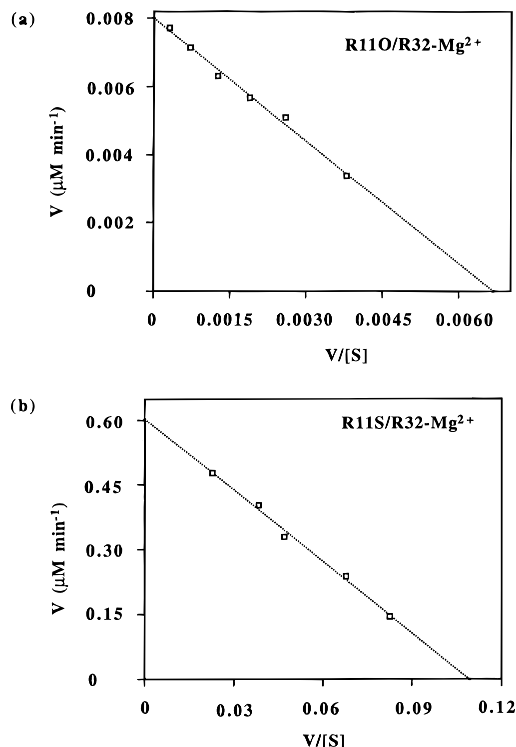

Figure 4. Kinetics of catalytic cleavage. (a) Eadie−Hofstee plot of kinetic data obtained from reactions in 50 mM MES buffer (pH 6.0) with 0.3 mM Mg^{2+} at 28 °C. Initial rates of cleavage were determined with 2.0 μM R32 and 2.0−25 μM R11O. The line yields a K_m of 1.2 μM and a V_{max} of 0.008 μM min^{-1}. (b) Eadie−Hofstee plot of kinetic data obtained under similar conditions to those of Figure 4a except that R11S was used instead of R11O as the substrate. The line yields a K_m of 5.5 μM and a V_{max} of 0.60 μM min^{-1}.

from the 3′ end, to yield 9-mer and 2-mer products. These products were analyzed by sequencing (by enzymatic and non-enzymatic hydrolysis). The latter cleavage occurred at a regular phosphodiester bond and we do not know, at present, how or why this bond became so labile. Although the 9-mer product (5′-GCCGUC$_{(S)}$CC-3′) could serve as a substrate for the ribozymes used in this study (R32 and R31), its K_m was found to be more than 20-fold higher and its k_{cat} was more than 10-fold lower than the corresponding values for the normal 11-mer substrate. Therefore, the 9-mer product did not act as a substrate nor as an inhibitor during the measurement of ribozyme-mediated cleavage of R11S (Figure 3). Even though the 9-mer product (about 20%; indicated by asterisk on the autoradiograms in Figure 3) was always observed, its concentration remained constant during the kinetic measurements. Therefore, we carried out all our kinetic measurements in the presence of these cleaved products (in practice, we could not avoid such contamination).

Results and Discussion

Since R11S was very labile, we had to identify reaction conditions under which non-ribozyme-mediated hydrolysis could be minimized. Thus, reactions were carried out at pH 6.0 and concentrations of Mg^{2+} ions were kept at 0.3 mM. Higher concentrations of Mg^{2+} ions (for example, 5 mM) produced approximately 60% cleavage products within a short time upon addition of metals. The rates of background reactions at pH 6.0 and with 0.3 mM metal ions were low enough for our purposes: the half-life of R11S was calculated to be 320 h with 0.3 mM Mg^{2+} ions. Time courses for the ribozyme-catalyzed reactions are shown in Figure 3 for R11O and R11S. We confirmed that, within the time frame of the ribozyme-catalyzed reactions (20 min for R11S), no background hydrolysis had occurred. Nevertheless, for all kinetic measurements, inactive ribozymes [I-R32 (see Table 1); in which G_5 was mutated to A (ref 23)] were tested in parallel as controls in order to confirm that, in a solution that contained an active ribozyme, we were really monitoring ribozyme-catalyzed reactions.

Table 1. Kinetic Parameters for Ribozyme-Catalyzed Cleavage Reactions[a]

sub/Rz	k_{cat}, min^{-1}	K_m, μM	k_{cat}(R11O)/ k_{cat}(R11S)	k_{cat}(R32)/ k_{cat}(R31)
R11O/R32	0.0040	1.2	85	
R11S/R32	0.34	4.8		45
R11S/R31	0.0075	7.3		
R11S/I-R32	0[b]			

[a] All measurements were made in the presence of 0.3 mM metal ions (M), 50 mM MES (pH 6.0) at 28 °C. [b] No cleavage of substrates (Sub) was observed within 20 min of the start of the reaction used to determine kinetic parameters for active ribozymes (Rsz: R32 and R31) at different concentrations of substrates. The inactive ribozyme (I-R32) differes from R32 by a single mutation of G_5 to A.

From our previously published Arrhenius plot,[24] we were able to predict that the k_{cat} in the present measurements would represent the rate of the chemical cleavage step (k_{cleav} in Figure 2 of ref 24). Thus, the rate of the product dissociation step (k_{diss}) is expected to be higher than the rate of the chemical cleavage step ($k_{cleav} < k_{diss}$).[24] Nevertheless, in order to confirm this prediction, under the conditions of our kinetic measurements at pH 6.0 with 0.3 mM metal ions, we used a 31-mer ribozyme (R31) that lacks one nucleotide at the 3′-end, as compared with the parental 32-mer ribozyme (R32). Our previous experiments demonstrated that, when the chemical cleavage step (k_{cleav}) was the rate-limiting step, shortening of the binding arm resulted in a decrease in k_{cat} and an increase in K_m. By contrast, if the product dissociation step (k_{diss}) were the rate-limiting step, shortening of the binding arm would result in an increase in k_{cat}.[25] When R11S was treated with R31 in the presence of Mg^{2+} ions, k_{cat} decreased 45-fold and K_m doubled, as compared to the values obtained with the parental R32 ribozyme (Table 1). Moreover, if the product dissociation step (k_{diss}) were the rate-limiting step, we would expect the identical k_{cat} for both R11O and R11S because, in both cases, cleavage products are nearly identical. Nevertheless, R11S underwent cleavage almost two orders of magnitude more rapidly than R11O. These results clearly prove that the k_{cat}, under the conditions of present kinetic measurements, represented the rate of the chemical cleavage step (k_{cleav}).

Kuimelis and McLaughlin concluded, from their analysis with the almost *all*-DNA substrate, that the attack by the 2′-oxygen at C_{17} on the phosphorus (**TS1**) is the rate-limiting step and that the departure of the 5′-leaving group (**TS2**) is not the rate-limiting step in the cleavage reaction catalyzed by a hammerhead ribozyme.[18] They reached this conclusion because the introduction of a 5′-bridging phosphorothioate linkage to the almost *all*-DNA substrate did not change the rate of the ribozyme-mediated cleavage. However, our observations are clearly different from theirs (Figure 3 and Table 1). In our case, R11S was cleaved by the ribozyme almost two orders of magnitude more rapidly than R11O in the presence of Mg^{2+} ions.

Of the two separate transition states designated **TS1** (attack by the 2′-oxygen on the phosphorus) and **TS2** (departure of the 5′-leaving group from the phosphorus), our molecular orbital calculations indicate that **TS2** is always a higher-energy state

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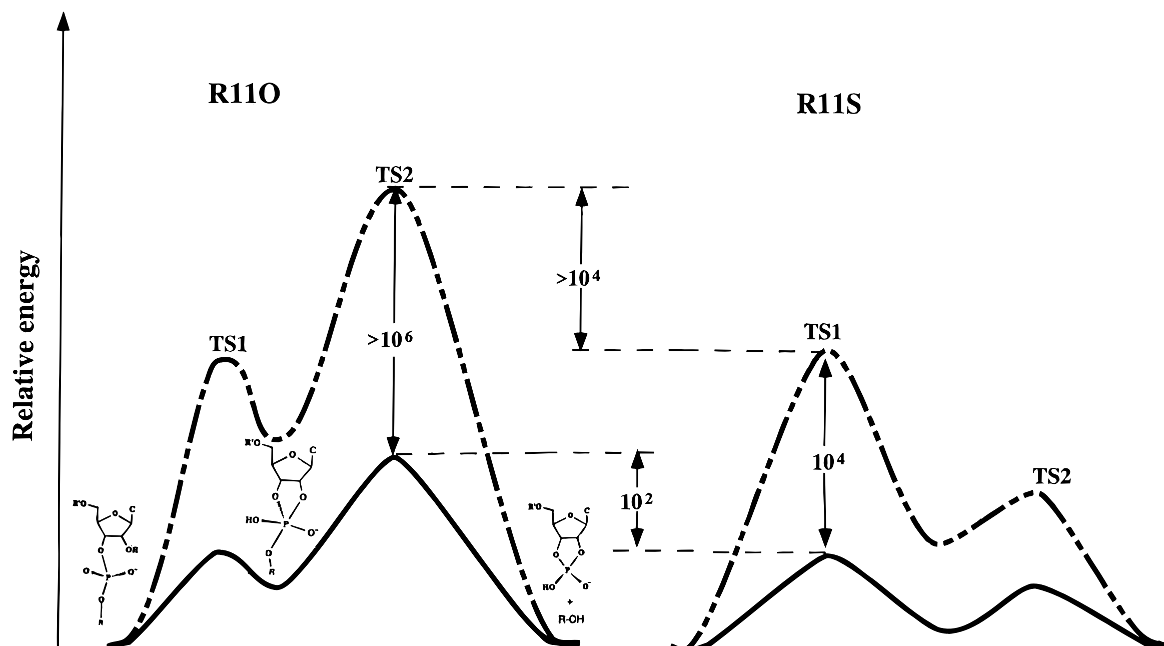

Figure 5. Relative energies, for the natural substrate (R11O; left) and a modified substrate with a 5′-bridging thiophosphate linkage (R11S; right), of reactions catalyzed by a hammerhead ribozyme (solid lines) as well as of non-enzymatic hydrolysis (dotted lines). The chemical cleavage step consists of the attack by the 2′-oxygen at C_{17} on the phosphorus (**TS1**) and the departure of the 5′-leaving group (**TS2**). Among the two separate transition states, **TS1** and **TS2**, according to our molecular orbital calculations,[26–32] **TS2** is always a higher-energy state than **TS1** in non-enzymatic hydrolysis (top left). This conclusion was further confirmed by the present analysis of the rates of non-enzymatic hydrolysis of R11O and R11S because R11S was more than four orders of magnitude more susceptible to cleavage than R11O. If the formation of the pentacoordinate intermediate [P(V)] were rate-limiting (if **TS1** were a higher energy state than **TS2**), R11S should have been hydrolyzed at a rate similar to the rate of hydrolysis of R11O because the 5′-bridging phosphorothioate linkage would not be expected to enhance the attack by 2′-oxygen.[37] Moreover, since the pK_a of a thiol is more than 5 units lower than that of an alcohol, **TS1** must be a higher energy state than **TS2** in the non-enzymatic hydrolysis of R11S (top right). The rate-limiting formation of **TS1** for R11S (top right) is more than four orders of magnitude faster than the rate-limiting formation of **TS2** for R11O (top left) in terms of the difference in rates of non-enzymatic reactions.[18,35] In contrast to the earlier conclusion,[18] the departure of the 5′-leaving group is also the rate-limiting step in the hammerhead ribozyme-catalyzed reaction with the natural substrate (bottom left).

than **TS1** in non-enzymatic hydrolysis;[26−32] the calculated energy diagram is similar to the top left energy diagram shown in Figure 5 that is drawn based on experimental measurements. We estimate the half-life of R11O at pH 6.0 to be >1000 years.[33,34] Since the half-life for the R11O•R32 complex at pH 6.0 in the presence of 0.3 mM Mg^{2+} ions is 170 min, the hammerhead ribozyme (R32) enhances the rate of hydrolysis of R11O by $>10^6$-fold (bottom left diagram in Figure 5). Rates of hydrolysis of R11S were measured in the absence and presence of the hammerhead ribozyme. At pH 6.0, in the presence of 0.3 mM Mg^{2+} ions, R11S was hydrolyzed at a rate of 3.7×10^{-5} min^{-1} and the corresponding R11S•R32 complex was hydrolyzed at a rate of 0.34 min^{-1}. Therefore, in the case of R11S, the hammerhead ribozyme (R32) enhanced the rate of hydrolysis 10^4-fold (bottom right diagram in Figure 5). Recently published data indicated that the rate of the rate-limiting formation of **TS1** for R11S (top right diagram) is at least four orders of magnitude lower than the rate of the rate-limiting formation of **TS2** for R11O (top left diagram) in terms of the difference in rates of non-enzymatic reactions.[18,35]

If the formation of the pentacoordinate intermediate was rate limiting (that is, if **TS1** was a higher energy state than **TS2**), R11S should have been hydrolyzed at a rate similar to the rate of the hydrolysis of R11O because the 5′-bridging phosphorothioate linkage would not be expected to enhance the attack by the 2′-oxygen.[36,37] By contrast, if the decomposition of the intermediate was rate limiting (that is, if **TS2** was a higher energy state than **TS1**), we would expect R11S to be hydrolyzed much more rapidly than R11O because the pK_a of thiol is more than 5 units lower than that of an alcohol.[38,39] Our results support the latter hypothesis in non-enzymatic hydrolysis of RNA (top left diagram in Figure 5).

Similarly, since the R11S•R32 complex was hydrolyzed almost two orders of magnitude more rapidly than the R11O•R32 complex, at pH 6.0 in the presence of 0.3 mM Mg^{2+} ions, the rate-limiting step cannot be the same in both cases. If the rate-limiting step were the formation of **TS1** in both cases, as suggested by Kuimelis and McLaughlin,[18] both R11O and R11S should have been hydrolyzed at the same rate. The discrepancy between Kuimelis and McLaughlin's result and ours could

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potentially originate from the differences in substrates used (almost *all*-DNA *versus all*-RNA), at least in part. Since we used natural *all*-RNA substrates and since our R11S was hydrolyzed by the ribozyme more than three orders of magnitude more rapidly than the corresponding DNA substrate of Kuimelis and McLaughlin (upon correction of pH and metal concentration to the same value), we believe that our result reflects true ribozyme-mediated catalysis. Therefore, the attack by the 2′-oxygen at C_{17} on the phosphorus (**TS1**) must be the rate-limiting step for R11S and the departure of the 5′-leaving group (**TS2**) must be the rate-limiting step for R11O, for the cleavage reactions catalyzed by a hammerhead ribozyme (as indicated by the bottom energy diagrams in Figure 5).

Since **TS2** is significantly stabilized (the pK_a of a thiol is more than 5 units lower than that of an alcohol), in the case of R11S it is likely that the 5′-thiol group leaves without the assistance of any metal ions. Note, moreover, that the hammerhead ribozyme enhances the rate of hydrolysis of the natural substrate (R11O) and R11S by a factor of $>10^6$ and 10^4, respectively, which nearly matches the extent of the stabilization gained by the good thio-leaving group ($>10^5$). Accordingly, we did not observe a switch in metal ion specificity: both the regular substrate (R11O) and the 5′-thio substrate (R11S) were cleaved more rapidly in a buffer that contained Mn^{2+} ions than in one that contained Mg^{2+} ions. We will make comments on this observation elsewhere.

In conclusion, we have been able to estimate, for the first time, the relative energies, for both a natural substrate and a modified substrate with a 5′-bridging thiophosphate linkage, of reactions catalyzed by a hammerhead ribozyme, as well as of non-enzymatic hydrolysis (Table 1 and Figure 5). In contrast to the earlier conclusion,[18] the departure of the 5′-leaving group is found to be the rate-limiting step in the hammerhead ribozyme-catalyzed reaction with the natural substrate. This conclusion does not depend on the lifetime of the pentacoordinate intermediate [P(V)]. If the lifetime of P(V) were kinetically insignificant (if the reaction were concerted), the actual, single transition state for R11O and R11S would be **TS2**-like and **TS1**-like, respectively.

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