Effects of substrate structure on the kinetics of circle opening reactions of the self-splicing intervening sequence from Tetrahymena thermophila: evidence for substrate and Mg$^{2+}$ binding interactions

Naoki Sugimoto, Mary Tomka, Ryszard Kierzek, Philip C. Bevilacqua and Douglas H. Turner*

Department of Chemistry, University of Rochester, Rochester, NY 14627, USA and 1Institute of Bioorganic Chemistry, Polish Academy of Science, 60-704 Poznan, Noskowskiego 12/14, Poland

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ABSTRACT
The self-splicing intervening sequence from the precursor rRNA of Tetrahymena thermophila cyclizes to form a covalently closed circle. This circle can be reopened by reaction with oligonucleotides or water. The kinetics of circle opening as a function of substrate and Mg$^{2+}$ concentrations have been measured for dCrU, rCdU, dCdT, and H$_2$O addition. Comparisons with previous results for rCrU suggest: (1) the 2'OH of the 5' sugar of a dinucleoside phosphate is involved in substrate binding, and (2) the 2'OH of the 3' sugar of a dimer substrate is involved in Mg$^{2+}$ binding. Evidently, the binding site for a required Mg$^{2+}$ ion is dependent on both the ribozyme and the dimer substrate.

The apparent activation energy and entropy for circle opening by hydrolysis are 31 kcal/mol and 50 eu, respectively. The large, positive activation entropy suggests a partial unfolding of the ribozyme is required for reaction.

INTRODUCTION
The discovery of RNA catalysis has led to great interest in the mechanisms for such reactions (1-8). The circular form of the self-splicing intervening sequence from the rRNA precursor of Tetrahymena thermophila (CIVS) provides a convenient model system for kinetic studies (9-12). This covalently closed RNA circle can be opened by addition of oligonucleotides (9) or by hydrolysis (10,11). Previous studies have indicated that binding of oligonucleotide substrate is unusually strong (1,9,12) and that a weakly bound Mg$^{2+}$ ion may be required for reaction (12). Both substrate binding and metal ion participation are important, but incompletely understood, aspects of RNA catalysis (13-17). In this paper, we report the effects on circle opening of changing the substrate from rCrU to dCrU, rCdU, dCdT, or H$_2$O. The results suggest the 5' sugar of substrate is involved in oligonucleotide binding, and the 3' sugar is involved in Mg$^{2+}$ binding.

MATERIALS AND METHODS
Nucleic Acids. dCdT was obtained from Sigma and purity confirmed by high performance liquid chromatography. dCrU and rCdU were synthesized on solid
support with a phosphoramidite method (18) and purified by high performance liquid chromatography (19). Concentrations were determined optically with extinction coefficients of $1.52 \times 10^4$, $1.73 \times 10^4$, and $1.59 \times 10^4$ M$^{-1}$cm$^{-1}$ for dCdT, dCrU, and rCdU, respectively (20).

C IVS was obtained by transcribing the plasmid pTIA3-T7 (3,21) with T7 RNA polymerase (22), and reacting the product as described previously (12). Labelled C IVS was obtained by transcribing in the presence of [alpha-$^{32}$P] ATP. C IVS was purified by running the reaction mixture on a 4% polyacrylamide/8 M urea gel and eluting the appropriate ethidium stained band. Final purification was by chromatography on Sephadex G-50. The concentration of unlabelled C IVS was determined optically with an extinction coefficient of $3.2 \times 10^4$ M$^{-1}$cm$^{-1}$ at 260 nm (23).

Reactions. Reactions were run in 20 µL volumes sealed in 40 µL glass capillaries (12). The buffer for circle opening by oligonucleotides was 10 mM NaCl, 0.5 mM EDTA, 10 mM PIPES, pH 6.5. The buffer for circle opening by hydrolysis was 10 mM NaCl, 50 mM HEPPS, adjusted to the appropriate pH. In both cases, various concentrations of Mg$^{2+}$ were added. To ensure pseudo-first-order conditions, the concentration of $^{32}$P labelled C IVS was about $10^{-8}$ M, always much less than the concentrations of oligomer and Mg$^{2+}$. Reactions were stopped by placing on ice and adding EDTA to a final concentration at least 2.5 times the magnesium concentration. Reaction mixtures were analyzed by running on 4% polyacrylamide/8 M urea gels, cutting out bands from dried gels, and quantifying radioactivity by scintillation counting (9,12).

Data Analysis. Extent of reaction was defined as either $[XY-L'IVS]/([XY-L'IVS]+[C IVS])$ or $[L'IVS]/([L'IVS]+[C IVS])$, where XY-L'IVS represents C IVS opened by dinucleoside monophosphate and L'IVS represents C IVS opened by hydrolysis. Extent of reaction vs. time points were analyzed with program DISCRETE, written by Provencher (24). In all cases, the single exponential fit was excellent. Non-linear least squares fits of observed rate constants to kinetic equations were done with a program written by L. Friedrich (Eastman Kodak Company).

RESULTS

Results for Dinucleoside Monophosphate Substrates. Typical time courses for the reaction of C IVS with rCdU at 30 °C in 10mM free Mg$^{2+}$, are shown in Figure 1. Time courses for dCrU and dCdT were similar in shape. It has been demonstrated that in the presence of rCrU, circle opening by OH$^-$ is suppressed (12). Thus all circle opening is essentially due to reaction with rCrU. This
Figure 1. a) Autoradiogram of denaturing 4% polyacrylamide gel showing time dependence of reverse cyclization by rCdU. Circular IVS was incubated at 30 °C with 1.0 mM rCdU in 10 mM NaCl, 10.5 mM MgCl₂, 0.5 mM EDTA, 10 mM PIPES, pH 6.5. From left to right, incubation times are 0,1,2,4,7,10,15,20,30,60h. b) Extent of reverse cyclization at 30 °C vs. time for the following concentrations of rCdU: (●) 0.01 mM, (○) 0.05 mM, (▲) 0.10 mM, (▲) 0.50 mM, (●) 1.00 mM. Points were corrected for linear species present at t=0, so that the extent of reaction is 0 at t=0 for each concentration. Buffer was 10 mM NaCl, 10.5 mM MgCl₂, 0.5 mM EDTA, 10 mM PIPES, pH 6.5. Each solid curve is a non-linear least squares fit of data points to the single exponential function of eq 1. Not shown for 0.05 mM rCdU is the point for 120 hours which had an extent of reaction of 38.1%.
also appears to be true for rCdU, dCrU, and dCdT, since the extent of reaction levels off at long times considerably below the 90% level observed for reaction with OH\(^-\) (see below). This was further tested by incubating 10\(^{-8}\) M dCdT labelled with \(^{32}\)P on the 5' end with 7.8 \(\times\) 10\(^{-5}\) M unlabelled C IVS for 120 hr under the conditions listed in the legend to Figure 1. The extent of reaction was determined by running the reaction mixture and an aliquot containing an equal amount of labelled dCdT on separate 4% polyacrylamide gels, cutting out and counting the dCdT-L'IVS and dCdT bands. Thirteen % of the labelled dCdT reacted to give labelled dCdT-L'IVS, implying an equilibrium constant of 1.9 \(\times\) 10\(^3\) M\(^{-1}\) for circle opening. This compares well with the equilibrium constant of 1.8 \(\times\) 10\(^3\) M\(^{-1}\) calculated from the 31% extent of reaction measured with 2.5 \(\times\) 10\(^{-4}\) M unlabelled dCdT and 10\(^{-8}\) M labelled C IVS. This agreement indicates essentially all the circle opening is due to reaction with dCdT. It is assumed this is also true for reaction with dCrU and rCdU.

The time courses for the fraction of linear form, \([\text{XY-L'IVS}] / [\text{XY-L'IVS}] + [\text{C IVS}]\), are represented well by a single exponential:

\[
\frac{[\text{XY-L'IVS}]}{[\text{XY-L'IVS}]+[\text{C IVS}]} = \left( \frac{[\text{XY-L'IVS}]}{[\text{XY-L'IVS}]+[\text{C IVS}]} \right)_{t=\infty} (1-e^{-k_{obs} t})
\]  

Curves fit to eq 1 using the method of Provencher (24) are also shown in Figure 1. Plots of the observed first order rate constant, \(k_{obs}\), as a function of the initial concentration of oligomer, \([\text{XY}]_0\), are shown in Figure 2. The observed rate constants reach limiting values at high dimer concentrations. Higher dimer concentrations are required to reach saturation with dCrU and dCdT than with rCrU and rCdU.

The effect of Mg\(^{2+}\) on the kinetics was determined by measuring \(k_{obs}\) as a function of [Mg\(^{2+}\)] at saturating concentrations of dimer, 1 mM for rCdU (and rCrU) and 3 mM for dCrU and dCdT. The results are shown in Figure 3. Evidently, the dimers with deoxyribose on the 3' end require higher Mg\(^{2+}\) concentrations to react with a half maximal \(k_{obs}\). Moreover, the maximum \(k_{obs}\) for dimers with a 3' terminal deoxyribose is about 5 times smaller than that observed with a 3' terminal ribose. In contrast, changing the 5' sugar has no effect on either the maximum \(k_{obs}\) or on the Mg\(^{2+}\) concentration required for saturation.

**Hydrolysis Reaction.** Typical time courses for circle opening by hydrolysis at 42°C in 10 mM NaCl, 50 mM HEPPS, pH 8, with various concentrations of
Figure 2. Plot of $k_{\text{obs}}$ vs. concentration of oligomer at 30 °C in the presence of 10 mM free $\text{Mg}^{2+}$. The oligomers are (■) dCdT, (○) rCdU, (▲) dCrU, and (▲) rCrU. Buffer was 10 mM NaCl, 0.5 mM EDTA, 10 mM PIPES, pH 6.5. Solid lines are non-linear least squares fits to eq 3.

$\text{Mg}^{2+}$ are shown in Figure 4. The time courses are well represented by eq 1 with XY-L'IVS replaced by L'IVS. Curves fit with the method of Provencher (24) are also shown in Figure 4. Plots of $k_{\text{obs}}$ vs. [Mg$^{2+}$] are shown in Figure 5 for both 42 and 30 °C. Comparison with Figure 3 indicates higher Mg$^{2+}$ concentrations are required to saturate the rate for hydrolysis than for oligomer substrate. Rates were also measured as a function of pH at 100 mM Mg$^{2+}$, 30 °C, and the results are shown in Figure 6. An Arrhenius plot of ln $k_{\text{obs}}$ vs 1/T at 200 mM Mg$^{2+}$ is shown in Figure 7. The activation energy derived from $k_{\text{obs}} = A \exp(-E_a/RT)$ is 31 kcal/mol. A similar activation energy is obtained if results at 100 mM Mg$^{2+}$ are plotted.
Figure 3. Plot of $k_{\text{obs}}$ vs. concentration of free Mg$^{2+}$ for circle opening by oligomer at saturating concentration at 30 °C. Buffer was 10 mM NaCl, 0.5 mM EDTA, 10 mM PIPES, pH 6.5. Figure symbols are the same as Figure 2. Solid lines are non-linear least squares fit to eq 5.

DISCUSSION

The results imply a non-covalent dimer-C IVS intermediate. The dependence of $k_{\text{obs}}$ on dimer concentration is consistent with the mechanism previously proposed for circle opening by rCrU (12):

$$
C \text{ IVS} + XY \xrightleftharpoons{k_1}{k_2} \text{ C IVS+XY} \xrightleftharpoons{k_{-1}}{k_{-2}} \text{ XY-L'IVS}
$$

(2)

Here C IVS+XY is a noncovalent complex of circle with oligomer. When the
Figure 4. Extent of hydrolysis at 42 °C vs. time in the presence of free Mg\(^{2+}\) at (Δ) 10 mM, (□) 50 mM, (Φ) 100 mM, and (▲) 200 mM. Points were corrected for linear species present at t=0, so that extent of reaction is 0 at t=0 for each concentration. Buffer was 10 mM NaCl, 50 mM HEPPS, pH 8.0. Solid curve is non-linear least squares fit of data points to a single exponential function. The fit for 200 mM Mg\(^{2+}\) is not shown because it is very similar to the fit for 100 mM Mg\(^{2+}\). Not shown for 10 mM Mg\(^{2+}\) are 45 and 60 hr. points for which the extent of reaction was 87% and 89%, respectively.

The first step is much faster than the second step, and [XY]_0 \(\gg\) [IVS], \(k_{ob5}\) is given by (12):

\[
k_{obs} = \frac{k_2 [XY]_0}{[XY]_0 + \frac{1}{K_1}} + k_{-2}
\]  

(3)

Here [XY]_0 is the initial concentration of XY, and \(K_1 = k_1/k_{-1}\). Non-linear least squares fits to eq 3 of the data shown in Figure 2 provide values for \(K_1\)
Figure 5. Plots of $k_{\text{obs}}$ vs. concentration of Mg$^{2+}$ for hydrolysis at 30 °C (■) and 42 °C (○) in 10 mM NaCl, 50 mM HEPPS, pH 8.0. Solid lines are non-linear least squares fits to eq. 7.

and $k_2$. These are listed in Table I, along with values determined previously for rCrU. Fitted curves are shown in Figure 2.

The value of $k_{-2}$ was too small to determine. In principle, $k_{-2}$ could be determined from $K_1$, $k_2$, and the overall equilibrium constant, $K_{\text{eq}}$. Unfortunately, $K_{\text{eq}}$ derived from the extent of reaction increases as oligomer concentration decreases. This suggests some of the C IVS may not be reactive or that additional interactions are present. Similar effects have been observed previously (9,12). The kinetic results should be relatively insensitive to this effect since oligomer is in large excess.

The 2' OH of the 5' nucleoside affects dimer binding. A striking conclusion from Table I is that the binding constant for dimer substrate depends on the 5' sugar, but not on the 3' sugar. Thus rCrU and rCdU bind with $K_1$'s of about $2 \times 10^4$ M$^{-1}$, whereas dCrU and dCdT bind with $K_1$'s of about $1 \times 10^3$
Figure 6. Plot of log $k_{\text{obs}}$ vs. pH for hydrolysis at 30 °C. Buffer was 10 mM NaCl, 200 mM MgCl$_2$, 50 mM HEPES. The solid line is a linear least squares fit of data points.

$M^{-1}$. Assuming U and T behave similarly, both comparisons suggest the 2' OH of rC contributes a free energy increment, $\Delta AG^0$, of about $-RT \ln 20 = -1.8$ kcal/mol to the binding energy. Presumably, this results from formation of a hydrogen bond. The value of -1.8 kcal/mol is consistent with the free energy increment recently predicted for hydrogen bond formation between base pairs in the absence of competing stacking interactions (25). It is also similar to the free energy increment measured from the kinetics of self-splicing upon removal of hydrogen bonding groups from the guanosine cofactor (13,14). It has been shown that the $\Delta AG^0$ for rCrU binding to C IVS is 5 to 6 kcal/mol more favorable than predicted for duplex formation involving the expected pairing and stacking interactions (12). The present results indicate the 2' OH of C is responsible for almost 2 kcal/mol of this extra free energy increment, but that the 2' OH of U makes no contribution. There are several possibilities
Figure 7. Arrhenius plot of $\ln k_{\text{obs}}$ vs. $1/T$ for hydrolysis reaction. The solid line is a linear least squares fit to the data. The derived value for $E_a$ is 31 kcal/mol.

for the unaccounted for 3 to 4 kcal/mol of binding energy. These include a potential base triple involving the GU pair (26), a hydrogen bond to the phosphate of G, and an unusual stacking interaction on the U.

The results are consistent with uptake of $\text{Mg}^{2+}$ by the dimer-C IVS intermediate. The dependence of $k_{\text{obs}}$ on $[\text{Mg}^{2+}]$ is consistent with the previously postulated uptake of $\text{Mg}^{2+}$ in the reaction mechanism (12):

$$C \text{ IVS} \cdot XY + \text{Mg}^{2+} \xrightarrow{k_a} \text{Mg}^{2+} \cdot \text{C IVS} \cdot XY \xrightarrow{k_b} \text{XY-L' IVS} \cdot \text{Mg}^{2+}$$

When $[\text{Mg}^{2+}] > [\text{C IVS}]$, $k_{\text{obs}}$ for this mechanism is given by (12):

$$k_{\text{obs}} = \frac{k_b [\text{Mg}^{2+}]_o}{[\text{Mg}^{2+}]_o + \frac{1}{K_a}} + k_b$$

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TABLE I.
Kinetic Constants for Circle Opening by Dinucleoside Monophosphates at 30°C

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>$[\text{Mg}^{2+}]_0$ (mM)</th>
<th>$K_a$ ($\times 10^{-3}$)</th>
<th>$K_b$ ($\times 10^{-3}$)</th>
<th>$k_1$ (h$^{-1}$)</th>
<th>$k_2$ (h$^{-1}$)</th>
<th>$[XY]_s$ (mM)</th>
<th>$K_a$ (M$^{-1}$)</th>
<th>$K_b$ (M$^{-1}$)</th>
<th>$k_{-b}$ (h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rCrU$^a$</td>
<td>10</td>
<td>25±4</td>
<td>0.50±0.03</td>
<td>b</td>
<td>1</td>
<td>510±260</td>
<td>0.51±0.06</td>
<td>0.07±0.02</td>
<td></td>
</tr>
<tr>
<td>dCrU</td>
<td>10</td>
<td>1.3±0.6</td>
<td>0.68±0.06</td>
<td>b</td>
<td>3</td>
<td>400±120</td>
<td>0.62±0.03</td>
<td>b</td>
<td></td>
</tr>
<tr>
<td>rCdU</td>
<td>10</td>
<td>18±9</td>
<td>0.08±0.01</td>
<td>b</td>
<td>1</td>
<td>56±30</td>
<td>0.14±0.04</td>
<td>0.02±0.04</td>
<td></td>
</tr>
<tr>
<td>dCdT</td>
<td>10</td>
<td>0.9±0.5</td>
<td>0.11±0.01</td>
<td>b</td>
<td>3</td>
<td>81±30</td>
<td>0.15±0.02</td>
<td>0.015±0.01</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Too small to be determined accurately.

Here $[\text{Mg}^{2+}]_0$ is the initial concentration of free Mg$^{2+}$, and $K_a = k_a / k_{-a}$. Non-linear least squares fits of the data give values for $K_a$, $K_b$, and $k_{-b}$. These are listed in Table I, along with values determined previously for rCrU.

Fitted curves are shown in Figure 3. Comparison of eqs 2 and 4, and the values in Table I suggest $k_b$ and $k_2$ are for the same reaction step or combination of steps.

The 2' OH of the 3' nucleoside affects Mg$^{2+}$ binding. A striking conclusion from the Mg$^{2+}$ dependence is that the binding constant for Mg$^{2+}$, $K_a$, depends on the 3' sugar, but not on the 5' sugar of the substrate. Thus $K_a$ is about 500 and 400 M$^{-1}$ for rCrU and dCrU, respectively, and about 60 and 80 M$^{-1}$ for rCdU and dCdT, respectively. Assuming U and T behave similarly, both comparisons suggest the 2' OH of the 3' nucleotide is involved in binding Mg$^{2+}$. The results in Table I indicate the 2' OH of the 3' nucleotide also affects another step of the reaction since $k_2$ and $k_b$ are both about 5 times faster when the 3' nucleotide is ribose, rather than deoxyribose. Activation parameters measured for reaction with rCrU suggest this second step is actually a combination of steps (12). Thus it is not yet possible to identify the likely origin of this effect.

Hydrolysis Reaction. For circle opening by hydrolysis, Zaug et al. (11) reported a linear dependence of log $k_{obs}$ on pH, consistent with a bimolecular reaction mechanism involving attack by OH$^-$. A bimolecular mechanism is reasonable for OH$^-$ since it is difficult to envision a strong binding site for OH$^-$ on RNA. The results in Figure 6 are consistent with this previous work. The saturation of $k_{obs}$ as Mg$^{2+}$ concentration increases (see Figure 5),

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however, suggests an additional step in which C IVS binds Mg$^{2+}$:

$$C \text{ IVS} + \text{Mg}^{2+} \xrightarrow{k_a} C \text{ IVS-Mg}^{2+}$$

(6)

$$C \text{ IVS-Mg}^{2+} + \text{OH}^- \xrightarrow{k_B} \text{L'IVS-Mg}^{2+}$$

For this mechanism, $k_{\text{obs}}$ is given by:

$$k_{\text{obs}} = \frac{k_B [\text{Mg}^{2+}]_o [\text{OH}^-]_o}{[\text{Mg}^{2+}]_o + K^{-\alpha}} + k^{-\beta}$$

(7)

Here $[\text{Mg}^{2+}]_o$ and $[\text{OH}^-]_o$ are the initial concentrations of Mg$^{2+}$ and OH$^-$, and $K = k^{-\alpha} / k^{-\beta}$. $[\text{OH}^-]$ was calculated with the temperature dependent dissociation constant of water, $K_w (27)$. Non-linear least squares fits of the data provide values for $K$ and $k_B$ at 30 and 42°C. These are listed in Table II. The values of $k^{-\beta}$ were too small to determine. The values for $K$ of 28 and 20 M$^{-1}$ at 30 and 42°C, respectively, indicate a small temperature dependence. This is common for binding of Mg$^{2+}$ to RNA (12,28-31). The absolute values, however, are ten to a hundred fold smaller than commonly observed for Mg$^{2+}$ binding to RNA. It has been suggested that such small affinities may reflect formation of specific, inner sphere complexes (12). Such specific binding may be necessary for a catalytically active Mg$^{2+}$.

The results in Table II can be compared with the measurements of Zaug et al. (11) by calculating the pseudo first order rate constant, $k = k_B [\text{Mg}^{2+}]_o / ([\text{Mg}^{2+}]_o + K^{-\alpha})$ at 42°C. For the 10 mM Mg$^{2+}$ concentration used by Zaug et al., the results in Table II give $k = 1600$ min$^{-1}$M$^{-1}$, similar to Zaug's value of 1700 min$^{-1}$M$^{-1}$.

Mechanistic Implications of Mg$^{2+}$ Dependence. It has been suggested from molecular orbital calculations (32) and kinetic results (12) that the mechanism for circle opening by rCrU may involve binding of a Mg$^{2+}$ in an inner sphere complex involving the ribose of U. This binding may occur after oligomer substrate is bound. The results in Tables I and II are consistent with this suggestion. The binding constant for Mg$^{2+}$ decreases when the 3' sugar is changed from ribose to deoxyribose. It decreases
TABLE II. Kinetic Constants for Circle Opening by Hydrolysis

<table>
<thead>
<tr>
<th>T (°C)</th>
<th>$K_a$ (M$^{-1}$)</th>
<th>$k_b$ $\times$ 10$^{-5}$ (h$^{-1}$ M$^{-1}$)</th>
<th>$E_a$ = 31 kcal/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>28±15</td>
<td>1.35±0.2</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>20±4</td>
<td>5.74±0.3</td>
<td>$\Delta S^a$ = 50 eu</td>
</tr>
</tbody>
</table>

Further when the substrate is OH$^-$. These trends are expected for a Mg$^{2+}$ binding site partially composed of the 3' ribose. If the Mg$^{2+}$ site was contained completely within C IVS, then less variation with substrate is expected. It is also unlikely these trends are due to an effect of Mg$^{2+}$ concentration on the conformation of C IVS, since this would require a different C IVS conformation for each substrate.

The value of $K_a$ observed for circle opening by OH$^-$ may represent the affinity of C IVS alone for the catalytic Mg$^{2+}$. This suggests the reaction mechanism may not be strictly ordered as in eq 4, but may involve random addition of oligomer and Mg$^{2+}$:

$$
\text{C IVS} \xrightarrow{K_a} \text{C IVS-Mg}^{2+} \rightleftharpoons \text{XY-C IVS} \xrightarrow{K_a} \text{XY-C IVS-Mg}^{2+} \rightleftharpoons \text{XY-L'IVS-Mg}^{2+}
$$

For this mechanism, the values of $K_a$ and $K_a$ refer to formation of the XY·C IVS·Mg$^{2+}$ intermediate if each is measured at saturating concentrations of the other substrate. The values of $K_a$ and $K_a$ indicate participation of a 3' ribose from the oligomer substrate increases the affinity for Mg$^{2+}$ by roughly a factor of 20.

In a previous study of circle opening by CU, the extent of reaction vs. time at 42 and 50 °C did not fit the single exponential of eq 1 (12). At least two exponentials were required. Both exponentials observed at 42 °C were faster than predicted by eq 7 for the hydrolysis reaction. Thus both exponentials likely arise from reaction with CU. At 35 °C, only a single exponential was observed. This indicates a second step in the mechanism becomes partially rate limiting above 35 °C. Evidently, many steps will be required to fully describe the mechanism for circle opening by oligomer.

Implications for Evolution and Molecular Recognition. Self-splicing RNA may represent a molecular fossil since it can both code information and have
catalytic activity (33-36). This suggests the participation of substrate in binding Mg$^{2+}$ may represent an early solution to the problem of developing a binding site for a catalytic metal ion. Such a binding site requires only limited evolution of the ribozyme. It also increases the specificity of reaction. In this model, the binding constant for Mg$^{2+}$ is about 30 M$^{-1}$ in the absence of RNA substrate and 500 M$^{-1}$ in the presence of substrate. Thus, at low Mg$^{2+}$ concentration, the Mg$^{2+}$ site is usually vacant until substrate binds. At subsaturating oligomer concentration, the concentration of occupied, catalytic Mg$^{2+}$ sites is related to the binding constant for RNA substrate. The Mg$^{2+}$ affinity may also be modulated by local structure dependent on appropriate pairing with substrate. Both effects would discriminate against improper substrates. A somewhat analogous approach has been used for designing small organic complexes for molecular recognition and catalysis (37).

The results of this study have another interesting implication for evolution. Reaction is observed with the substrate dCdT. Previously, dC$_5$ has been shown to be a substrate in a related reaction (see caption of Figure 5 in reference 3). Thus ribozymes are able to catalyze reactions of oligodeoxynucleotides as well as oligoribonucleotides. This could have facilitated conversion from RNA to DNA as the carrier of genetic information.

Comparison with Mg$^{2+}$ Dependence for other RNA Catalyzed Reactions. There is only limited data on the Mg$^{2+}$ dependence of the kinetics for RNA catalyzed reactions. Pace and coworkers (17) have measured rates for cleavage of tRNA precursor by the RNA component of RNase P from Bacillus subtilis. They find that increasing [Mg$^{2+}$] does not affect the catalytic rate, but does affect the binding constant for substrate to ribozyme. Thus the Mg$^{2+}$ dependence for cleavage of tRNA precursors is quite different from that for circle opening. Uhlenbeck (8) has measured the Mg$^{2+}$ dependence for the rate of cleavage in an oligonucleotide model for self-cleaving viroids (7). The data suggest rate saturation above 20 mM Mg$^{2+}$. This is consistent with the type of Mg$^{2+}$ binding proposed here for circle opening. Alternative origins are also possible, however. Clearly, more work is required to determine all the roles of Mg$^{2+}$ ions in RNA catalysis.

Activation Entropy for Hydrolysis. An activation entropy can be calculated for hydrolysis by using the measured activation energy and rate for saturating concentrations of Mg$^{2+}$. The fit of the 30 °C data in Figure 5 to eq 7 gives a rate at saturation of $k_{obs}$ = $k_p [OH^-]$ = 0.2 h$^{-1}$. The OH$^-$ concentration for this case is 1.5 X 10$^{-6}$ M, giving $k_p$ = 37 M$^{-1}$ s$^{-1}$. For the mechanism of eq 6, the measured $E_a$ of 31 kcal/mol at saturating Mg$^{2+}$ is the activation energy
for $k_\beta$ because $k_{-\beta}$ is small (see eq 7). This gives an activation entropy, $\Delta S^*$, of 50 eu from the Eyring equation:

$$k = \frac{e^{RT}}{Nh} \exp\left(-\frac{E_a}{RT}\right) \exp\left(\frac{\Delta S^*/R}{a}\right)$$  \hspace{1cm} (9)$$

Here $e$ is the base of natural logarithms (2.72), $N$ is Avogadro's number, and $h$ is Planck's constant.

The activation entropy measured for circle opening by hydrolysis can be compared with that reported for circle opening by rCrU (12). The $\Delta S^*$ is large and positive in both cases, 50 and 86 eu, respectively, for hydrolysis and rCrU. This is unusual and suggests the second steps in eq 2, 4 and 6 are actually more than one step. Thus the differences in activation parameters are hard to interpret in detail. Nevertheless, the relative magnitudes appear consistent with the mechanisms proposed in eq 4 and 6. The activation entropy represents the difference in entropy between the activated complex for the rate determining step and the intermediates shown in eq 4 and 6. If the activated complexes for the rate determining step for reaction with rCrU and OH$^-$ are similar, then the differences in activation entropy would depend on the differences in the intermediates. The intermediate for reaction with rCrU, Mg$^{2+}$·C IVS·CU is a single species containing all three reaction components. The intermediate for hydrolysis, Mg$^{2+}$·C IVS, does not contain the OH$^-$ substrate. Thus the reaction components are likely to be more ordered in the rCrU intermediate than in the hydrolysis intermediate. Since $\Delta S^*$ is positive, formation of the activated complex requires dis-ordering of this intermediate. Because the intermediate with rCrU is more ordered, $\Delta S^*$ for rCrU should be more positive than for OH$^-$. This is consistent with the measured values. A similar argument can be made for the activation energies.

The implication that the intermediate is more ordered than the transition state is unusual. The results suggest a partial unfolding of C IVS is required for reaction. The relative magnitudes of the activation parameters are consistent with a similar unfolding of C IVS during hydrolysis and reaction with rCrU.

SUMMARY

The kinetic effects of changing the substrate for circle opening from rCrU to dCrU, rCdU, dCdT, or OH$^-$ have been measured. The results suggest a hydrogen bond between C IVS and the 2' OH of the 5' sugar of dimer substrate increases the binding constant between the two. The results are also consis-
tent with the 2' OH of the 3' sugar of a dimer substrate being involved in binding a Mg$^{2+}$ ion required for accelerating reaction. The activation parameters for hydrolysis are consistent with a partial unfolding of the C IVS structure during reaction.

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*To whom correspondence should be addressed

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