Origins of the temperature dependence of hammerhead ribozyme catalysis

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Received March 24, 1999; Revised and Accepted June 1, 1999

ABSTRACT

The difficulties in interpreting the temperature dependence of protein enzyme reactions are well recognized. Here, the hammerhead ribozyme cleavage was investigated under single-turnover conditions between 0 and 60°C as a model for RNA-catalyzed reactions. Under the adopted conditions, the chemical step appears to be rate-limiting. However, the observed rate of cleavage is affected by pre-catalytic equilibria involving deprotonation of an essential group and binding of at least one low-affinity Mg²⁺ ion. Thus, the apparent entropy and enthalpy of activation include contributions from the temperature dependence of these equilibria, precluding a simple physical interpretation of the observed activation parameters. Similar pre-catalytic equilibria likely contribute to the observed activation parameters for ribozyme reactions in general. The Arrhenius plot for the hammerhead reaction is substantially curved over the temperature range considered, which suggests the occurrence of a conformational change of the ribozyme ground state around physiological temperatures.

INTRODUCTION

According to transition state theory, analysis of the temperature dependence of a chemical reaction allows calculation of the activation parameters (enthalpy and entropy of activation) describing the thermodynamic differences between the ground state and the transition state of the reaction (1). In principle, knowledge of these differences may provide information on the nature of the transition state and on the reaction mechanism. For example, a substantial negative entropy of activation suggests the need for the reactants to properly orient themselves in the transition state, whereas a large enthalpy of activation hinders simple physical interpretations of the observed enthalpy dependence (e.g., 6–9). Thus, the temperature dependences of ribozyme reactions might be easier to study and to interpret than those of protein enzymes, and the observed activation parameters could shed some light on the properties of transition states in RNA catalysis. The hammerhead ribozyme (Fig. 1A; for reviews see 10–12) represents a particularly promising candidate for this kind of study. A kinetic and thermodynamic framework has been obtained for the hammerhead reaction (8), which allows the chemical step (Fig. 1B) to be unambiguously isolated, and the three-dimensional structure of the ground-state ribozyme–substrate complex has been solved at atomic resolution (13–15).

Whereas reporting the enthalpies and entropies of activation for the hammerhead reaction has been almost customary in the field (e.g., 16–20), the significance of these parameters and their relationship to the ribozyme mechanism has yet to be analyzed in detail. Furthermore, significant differences exist between the activation parameters reported in different studies. In some cases, these differences may reflect conformational problems of the adopted RNA constructs or uncertainties as to the reaction step that was being monitored (21).

In the present work, I describe the specific dependence of the cleavage step for a well-behaved hammerhead construct, under different sets of conditions and over a wide temperature range (0–60°C). The results of this work suggest some further explanations for the discrepancies between literature reports. More importantly, analysis of the results implies that a series of complexities integral to the hammerhead ribozyme mechanism hinder simple physical interpretations of the observed enthalpy and entropy of activation.

MATERIALS AND METHODS

Materials

The ribozyme used in this study, HH16, and its substrate were prepared by solid-phase synthesis (22) and generously provided by Dr L. Beigelman (Ribozyme Pharmaceuticals Inc., Boulder, CO). The ribozyme was purified by anion-exchange HPLC.
The substrate was $^{32}$P-5' end-labeled with T4 polynucleotide kinase and gel purified.

The following buffers from Sigma were used: 2-(N-morpholino)-ethanesulfonic acid (MES), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 1,3-bis[tris(hydroxymethyl)methylamino]propane (BisTris-propane) and tris(hydroxymethyl)aminomethane (Tris). The pH was adjusted directly at the temperature where measurements were to be performed, using NaOH (MES and HEPES) or HCl (Tris and BisTris-propane). For the MES and HEPES buffers, the final concentration of Na$^+$ and the ionic strength at each temperature range considered. The following buffers from Sigma were used: 2-(N-morpholino)-ethanesulfonic acid (MES), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 1,3-bis[tris(hydroxymethyl)methylamino]propane (BisTris-propane) and tris(hydroxymethyl)aminomethane (Tris). The pH was adjusted directly at the temperature where measurements were to be performed, using NaOH (MES and HEPES) or HCl (Tris and BisTris-propane). For the MES and HEPES buffers, the final concentration of Na$^+$ and the ionic strength at each temperature were also adjusted to a constant value (~100 mM before the addition of MgCl$_2$) by adding solid NaCl.

**Methods**

Previous studies of the hammerhead used in this study, HH16, have established conditions that allow the cleavage step of the ribozyme-substrate complex to be specifically followed (8). Reactions were single-turnover; the excess of ribozyme (typically 0.6 µM final concentration) with respect to substrate (~0.2 nM final concentration) ensured that the product dissociation step did not affect the observed kinetics. The reaction protocol was described previously (23). Briefly, substrate and ribozyme were mixed in an Eppendorf tube containing the desired buffer (50 mM final concentration). Tubes were heated at 95°C for 2 min to disrupt potential RNA aggregates, cooled to allow formation of the ribozyme–substrate complex and equilibrated for 10 min at the desired reaction temperature in a thermostated water bath (for measurements at 0°C, the bath contained a mixture of water and crushed ice). The temperature in the water baths was continuously monitored by a precision mercury thermometer and kept within 0.2°C from the desired value. Reactions were initiated by adding MgCl$_2$ at the desired final concentration (total volume of the reaction mixture: 30 µl). Time-points were collected at appropriate intervals and further reaction was quenched by adding formamide and EDTA. Products and substrates were separated on 7 M urea/20% polyacrylamide gels and their ratio was quantitated using a PhosphorImager (Molecular Dynamics). For each reaction, $k_{2obs}$ (the rate constant of cleavage of the ribozyme–substrate complex) was obtained by non-linear least-squares fitting the reaction time courses using Sigma Plot (Jandel Scientific). In all cases the data fit well to a simple exponential function ($R^2 > 0.99$); the end-points ranged between 70 and 85%, with the lower values observed at the lower temperatures.

In control reactions, increasing the ribozyme concentration from 0.6 to 2.5 µM did not change the observed reaction rate (Fig. 2A), confirming that substrate association was not rate limiting and implying that substrate was completely bound to the ribozyme over the entire temperature range explored in this study. As a further and more detailed control, the cleavage of substrate was measured as a function of [HH16] at 60°C, the highest temperature adopted in this study (conditions: ~0.2 nM substrate, 1–800 nM HH16, 50 mM MES·Na, pH 6.5, 10 mM Mg$^{2+}$). The observed $K_{d1/2}$ (i.e., the concentration of ribozyme required to achieve 50% of the maximum observed substrate cleavage rate) was 19 ± 5 nM. Nearest-neighbor calculations on the stability of helices I and III of HH16 (24), including the estimated energetic penalty for closure of the hammerhead core (8), predict a $K_d$ for the substrate of 30 nM at 60°C.

**RESULTS AND DISCUSSION**

The hammerhead reaction depends on pH and on [Mg$^{2+}$] over a wide temperature range

The rate of cleavage of the hammerhead ribozyme–substrate complex, measured at pH 6.5 and 10 mM Mg$^{2+}$, increased ~1000-fold between 0 and 60°C (Fig. 2A). The observed rate of cleavage was not significantly affected by the type of buffer used; however, $k_{2obs}$ values measured in buffers at pH 7.5 were about one order of magnitude higher than $k_{2obs}$ values measured at pH 6.5 (Fig. 2A). The pH-dependence of $k_{2obs}$ was maintained over the whole temperature range explored; even though reactions carried out at pH 7.5 and temperature >40°C were too fast for accurate measurement, the cleavage rate observed at pH 5.8, 60°C, was ~6-fold slower than at pH 6.5, 60°C (data not shown). As the rate of chemical cleavage in the hammerhead system is known to increase log-linearly with pH up to pH ~9 (25), these data strongly suggest that the chemical step of the hammerhead reaction is rate-limiting over the entire temperature range considered.
The dependence of the hammerhead catalysis on pH has been proposed to reflect the ionization of a metal-bound water molecule to form hydroxyde, which would act as a general base in the cleavage mechanism (25). Alternatively, the pH dependence may indicate that a secondary ionization event is directly involved in the catalytic mechanism of the ribozyme (30–32) or required to stabilize a fully active conformation, either by binding to some specific site or, less specifically, by increasing density of positive charges around the hammerhead structure (33–36). In any instance, the data in Figure 2B imply that the ribozyme–substrate complex needs to bind at least one additional Mg$^{2+}$ ion en route from the ground state to the transition state. This pre-catalytic metal association event, together with the pre-catalytic proton release described above, is represented schematically in Figure 2C.

Pre-catalytic equilibria affect the observed enthalpy and entropy of activation

In transition state theory, transition states can be treated as if they were in equilibrium with the respective ground states. According to a simple formulation of the theory, equations 1 and 2 relate the reaction rate constant, \( k \), to the difference in free energy, enthalpy and entropy between the ground state and the transition state:

\[
\ln(k) = \frac{-\Delta G^\ddagger}{RT} + \ln\left(\frac{k_B T}{h}\right)
\]

\[
\ln(k) = \frac{-\Delta H^\ddagger}{RT} + \frac{\Delta S^\ddagger}{R} + \ln\left(\frac{k_B T}{h}\right)
\]

\( \Delta G^\ddagger \) is the Gibbs free energy of activation; \( \Delta H^\ddagger \) and \( \Delta S^\ddagger \) are the enthalpy of activation and the entropy of activation, respectively; \( h \) is Planck’s constant, \( k_B \) is Boltzmann’s constant and \( T \) is the absolute temperature.

For the hammerhead cleavage reaction, the \( \Delta H^\ddagger \) values reported in different studies range from 15 to 25 kcal/mol and the \( \Delta S^\ddagger \) values range from \(-41\) to \(+17\) e.u. (16–20); in this study the \( \Delta H^\ddagger \) and \( \Delta S^\ddagger \) values for the cleavage process were estimated to be \(-30\) kcal/mol and \(+36\) e.u., respectively (at \( pH \) 7.5, 10 mM Mg$^{2+}$ and low temperature; see Fig. 3 and discussions below). It must be stressed however that all these values refer to apparent activation parameters, even in cases when the chemical step is certainly rate-limiting. Apparent activation parameters are influenced by rapid equilibrium processes occurring before the kinetic step that is being examined: for example, if a rate constant depends on the free base species

### Figure 1

(A) The temperature dependence of the hammerhead cleavage reaction in 10 mM Mg$^{2+}$ at \( pH \) 6.5 (open circles) and 7.5 (squares and diamonds) in MES·Na (open circles, open squares) or Tris·HCl (closed circles, closed squares). Crossed symbols refer to experiments where the concentration of ribozyme was 2.5 M instead of 0.6 M. (B) Temperature dependence of the hammerhead cleavage reaction in 100 mM Mg$^{2+}$, MES·Na \( pH \) 6.5 (closed triangles) compared to the temperature dependence of 100 mM Mg$^{2+}$, MES·Na \( pH \) 6.5 (closed circles) compared to the temperature dependence of 10 mM Mg$^{2+}$, same \( pH \) and buffer (open circles). (C) Pre-catalytic association–dissociation steps in the hammerhead mechanism. Under the conditions commonly adopted in hammerhead studies (10 mM Mg$^{2+}$, \( pH \) 7.5) passage from the ribozyme–substrate ground state to the transition state includes association of some metal ion (for simplicity, the model assumes binding of only one Mg$^{2+}$; this is not meant to exclude the involvement of multiple critical metal ions in hammerhead catalysis) and loss of a proton, as well as the chemical step proper. \( k_{\text{bind}}^{\text{Mg}} \) is the Mg$^{2+}$ association constant and \( k_{\text{diss}}^{\text{Mg}} \) is the proton dissociation constant. The overall rate of cleavage is described by \( k_{\text{cleav}} \) and represented by the dashed arrow. Inset: equations 3–5, derived from this simplified model, describe how the pre-catalytic steps influence \( k_{\text{cleav}} \) and therefore the apparent enthalpy and entropy of activation for the cleavage (\( \Delta H_{\text{cleav}}^\ddagger \) and \( \Delta S_{\text{cleav}}^\ddagger \), respectively). Derivation of these equations is given in the Appendix.
squares fitting of the individual sets of data to equation 7 were assumed constant (\( = 27 \text{ kcal/mol}, = 88.3 \text{ e.u.} \) and curve), 33.5 e.u. (middle curve) and 36.1 e.u. (upper curve). The other parameters of a thermodynamic represent the standard entropy of activation (i.e., the equivalent molar° fold difference in \([H^+]\). This 'concentration effect' underscores more positive by 4.6 e.u. than at pH 6.5, simply due to the 10-

example, the model and equation \( \Delta H^\ddagger \) and the case of the hammerhead reaction, the observed values of the effect of temperature on the dissociation equilibrium (1). In dependence of the reaction will have, as one of its components, Mg\(^{2+}\), MES·Na pH 6.5 (closed triangles) or in 10 mM Mg\(^{2+}\), HEPES·Na pH 7.5 (open diamonds). Solid lines through the data points were calculated using equation 7. Only \( \Delta S^\ddagger_{\text{conf}} \) was varied: \( \Delta S^\ddagger_{\text{conf}} \) values were 29.7 e.u. (lower curve), 33.5 e.u. (middle curve) and 36.1 e.u. (upper curve). The other parameters were assumed constant (\( \Delta H^\ddagger_{\text{conf}} = 27 \text{ kcal/mol}, \Delta S^0_{\text{conf}} = 88.3 \text{ e.u.} \) and \( \Delta H^\ddagger_{\text{cleav}} = 30.3 \text{ kcal/mol} \), and represent averages from the non-linear least-squares fitting of the individual sets of data to equation 7.

of a nucleophile at pH values below its \( pK_a \) the temperature dependence of the reaction will have, as one of its components, the effect of temperature on the dissociation equilibrium (1). In the case of the hammerhead reaction, the observed values of \( \Delta H^\ddagger \) and \( \Delta S^\ddagger \) will be influenced by the pre-catalytic equilibria shown in Figure 2C.

First, since the first-order rate constant for overall cleavage (indicated as \( k_{\text{cleav}} \) in Fig. 2C) varies with the concentration of Mg\(^{2+}\) and with pH, the apparent entropy of activation will also depend on the chosen concentrations of Mg\(^{2+}\) and protons. For example, the model and equation 5 in Figure 2C predict that, under otherwise identical conditions, \( \Delta S^\ddagger_{\text{cleav}} \) at pH 7.5 will be more positive by 4.6 e.u. than at pH 6.5, simply due to the 10-fold difference in \([H^+]\). This ‘concentration effect’ underscores some inherent problems in reporting entropy changes when the reaction is not purely unimolecular (1). In such cases, the apparent entropy of activation partly reflects the conventions followed in expressing the equilibrium and rate constants. In particular, if, as here, the rate constant does not account for the complex molecularity of a process, the apparent \( \Delta S^\ddagger \) will not represent the standard entropy of activation (i.e., the equivalent of a thermodynamic \( \Delta S^0 \)).

Second, both the observed entropy and enthalpy of activation will be affected by the temperature dependence of the pre-catalytic equilibrium constants. Since the exact nature of the pre-catalytic equilibria is not known, it is difficult to anticipate how they may contribute to the observed activation parameters. Deprotonation of most groups is an endothermic process: for example, the deprotonation of water is characterized by \( \Delta H^0 = +13.5 \text{ kcal/mol at 25°C (\( \Delta S^0 = -18.8 \text{ e.u.} \)) (37) while for the 2’-hydroxy of 5’-AMP, \( \Delta H^0 = +10.9 \text{ kcal/mol at the same temperature (\( \Delta S^0 = -23.3 \text{ e.u.} \) (26). Thus, the ionization equilibrium could contribute substantially to the observed enthalpy of activation; but another way, since the proton dissociation is expected to increase substantially with temperature, this might account for a large part of the rise in reaction rate between 0 and 60°C.

As for metal binding, inner-sphere coordination of Mg\(^{2+}\) to nucleotides 5’-monophosphates is an endothermic process (\( \Delta H^0 \approx +1.8 \text{ kcal/mol, } \Delta S^0 = +14 \text{ e.u.} \)) (38). A study of Mg\(^{2+}\) binding to simple RNA polymers (poly-A, poly-U and a poly-A/poly-U duplex) suggested that inner-sphere coordination of Mg\(^{2+}\) to RNA is also modestly endothermic; however, an exothermic, entropically unfavorable process was proposed if association of the metal with RNA involves only outer sphere interactions (39). In all the systems cited above, the changes in the standard enthalpy and entropy accompanying Mg\(^{2+}\) association are small; in the hammerhead ribozyme, however, more substantial \( \Delta H^\ddagger \) and \( \Delta S^\ddagger \) could arise from the coupling of metal binding and structural rearrangements, as it is known that Mg\(^{2+}\) can change the ribozyme conformation (33,34).

In conclusion, the presence of pre-catalytic association–dissociation equilibria contributes to the temperature dependence of catalysis by the hammerhead ribozyme. The extent of these contributions cannot be rigorously assessed, but it is likely substantial. The situation is complicated even further by the possible effects of temperature on the conformation of the ground-state ribozyme–substrate complex (see below). All of these complexities hinder a mechanistic interpretation of the observed activation parameters. More detailed information on the pre-catalytic equilibria will be required for a meaningful analysis of the apparent enthalpy and entropy of activation.

**Curved Arrhenius plots of the hammerhead reaction suggest a temperature-induced change of the ribozyme conformation**

Equation 2 predicts that a plot of the logarithm of the rate constant versus \( 1/T \) (the so-called Arrhenius plot) should be nearly linear around physiological temperatures, as the term \( \ln(k_B \cdot T/h) \) in equation 2 changes by <4% in the interval between 0 and 60°C. Instead, the Arrhenius plot of the hammerhead cleavage reaction shows a substantial deviation from linearity (Fig. 3).

In previous studies on the hammerhead ribozyme, Arrhenius plots curved at high temperature were tentatively attributed to dissociation of the ribozyme–substrate complex (16,40,41). However, the HH16 substrate was completely saturated by the ribozyme under all experimental conditions herein (see Materials and Methods). More generally, curvature could occur if the observed reaction involved two or more elementary processes with different activation enthalpies (16,42). In this case, however, chemistry appears to be rate limiting over the whole temperature range considered.

In the protein enzyme field, curved Arrhenius plots can often be ascribed to temperature-induced changes in the conformation of the catalyst (42). Analogously, it seems very plausible that the ground-state hammerhead conformation can change over a 60-degree interval. In the simplest scenario, high temperatures could stabilize an unproductive ground-state ribozyme–substrate complex (\( ES^\ddagger_{\text{conf}} \)), as depicted in Scheme 1.

If the interconversion between the two ribozyme conformations is fast compared to catalysis, equations 6 and 7 hold (see Appendix), where \( \Delta H^\ddagger_{\text{conf}} \) and \( \Delta S^0_{\text{conf}} \) are the standard
suggest that indeed this helix is still intact at 60°C known to be hyperstable (43) and RNase mapping experiments excluded: the particular stem–loop II in the HH16 construct is for the observed curvature? Unfolding of helix II can be

of the apparent curvature? Unfolding of helix II can be formally described as a substantial temperature dependence depending on the composition of this helix.

Other possible contributions to the curved Arrhenius plot: changes in heat capacity

The curvature in the Arrhenius plot of the hammerhead reaction can be formally described as a substantial temperature dependence of the apparent ΔH‡ and ΔS‡, i.e., as a substantial apparent heat capacity of activation (ΔCp‡). Fittings of the experimental data to a version of equation 2 that explicitly includes the heat capacity of activation (equation given in 47) suggest that a negative ΔCp‡ of −400 to −600 e.u. would be required to produce the observed curvature (not shown).

Model cleavage reactions in water are not are not accompanied by large ΔCp‡ values. For example, a detailed study on the non-catalytic hydrolysis of cytidine cyclic 2′3′ phosphate at different pH values did not find a significant heat capacity of activation for this reaction (48; for other examples see 49). This suggests that the large ΔCp‡ for the hammerhead reaction mirrors either the specific features of the ribozyme mechanism or the properties of the ribozyme structure. A large apparent ΔCp‡ can arise when the reaction ground state undergoes a temperature-controlled conformational shift (see discussions in 50)—this corresponds to the model in Scheme 1. Other factors that may account for the curved Arrhenius plot are discussed below.

A large ΔCp‡ might imply a large difference between the structure of the reaction ground state and that of the transition state (50). Indeed, there is evidence for a substantial rearrangement in the hammerhead structure preceding cleavage (23 and references therein) and it is known that conformational changes and folding events in proteins are often accompanied by large negative ΔCp values (51). However, the heat capacity changes in proteins arise mainly from large variations in solvent exposure of hydrophobic surfaces (51,52); such large variations are unlikely to accompany a tertiary rearrangement of the small hammerhead motif. Furthermore, compared to protein systems, structural transitions in RNA seem generally associated to much smaller ΔCp values (e.g., 53,54). Thus, a structural difference between the ground state and the transition state of the hammerhead reaction would not justify the observed large ΔCp‡.

Changes in heat capacity accompanying the pre-catalytic proton release and metal binding events could contribute to the overall curvature of the Arrhenius plot. However, ionization of small molecules, including water, is usually accompanied by only modest changes in standard heat capacity, on the order of 40 e.u. (55). The ΔCp‡ for Mg2+ binding to model ligands, such as glucose 1-phosphate or the phosphate trianion, is also small and usually positive (around +60 e.u.) (38). Accordingly, the contributions of the pre-catalytic equilibria depicted in Figure 2C to the curvature of the Arrhenius plots are probably minimal.

CONCLUSIONS

The rate of the hammerhead cleavage reaction, and therefore the temperature dependence of this reaction, comprises contributions from some critical association–dissociation pre-catalytic equilibria. This situation seems common in the field of RNA catalysis. For several well-characterized ribozymes the rate of the chemical reaction increases with pH (e.g., 56–58) and is affected by binding of multiple metal ions (e.g., 58–60), although saturation of catalysis is often achieved at Mg2+ concentrations lower than 100 mM. The presence of such functionally important equilibria is going to affect the observed activation parameters: for example, it has been noted that the entropy of activation for the Tetrahymena group I ribozyme reaction changes with pH and with [Mg2+] (61). This limits the
possibilities of providing simple physical interpretations of the apparent entropy and enthalpy of activation for RNA-catalyzed reactions in general.

A more profitable use of temperature dependences might lie in the analysis of differential activation parameters (ΔΔS, ΔΔH‡) obtained, for example, by comparing the effect of temperature on wild-type and mutant catalysts (62). An analogous approach has been followed in some ribozyme studies (e.g., 19, 41, 61, 63). The advantage of this approach is that it removes some ambiguities: when differential activation parameters are calculated several spurious contributions to the temperature dependence, including the concentration effects, cancel out (62). A limit is that, when a reaction rate depends on temperature dependence, including the concentration effects, any principle change the observed temperature dependence by affecting some pre-catalytic equilibrium, or the chemical step, or both. Thus, additional information is necessary while analyzing the differential activation parameters for mutants.

Temperature dependence studies can reveal curvatures or breaks in the Arrhenius plot of a reaction, which in turn may betray the existence of kinetic complexities in the process being studied or temperature-controlled changes in the reactants (16, 64, 65). The curved Arrhenius plot for the hammerhead reaction suggests that the ribozyme ground-state structure undergoes a temperature-induced change near physiological temperatures. This opens the way to further investigations, which may include the extension of the present analysis to other hammerhead constructs (see below) and the use of a combination of kinetic and structural techniques to clarify the molecular basis of the phenomenon.

The curvature in the Arrhenius plot also suggests a plausible explanation for some of the discrepancies between the activation parameters reported in different hammerhead studies. A plot of kinetic data collected over a modest temperature range (e.g., between 10 and 40°C; Fig. 3) would still look approximately linear and could be reasonably fit to equation 2 or to an equivalent linear equation); however, the apparent enthalpy and entropy of activation would depend on the choice of the temperature interval. The situation might be even more complicated because it is not clear whether the observed curvature is strictly conserved in all hammerhead systems. For example, if a rearrangement of helices I and III plays a role in determining the curved Arrhenius plot, hammerheads varying in the helices’ length or composition could display different curvatures, further favoring the variability in the apparent enthalpy and entropy of activation.

ACKNOWLEDGEMENTS

The experimental part of this work was carried out in the laboratory of Dr Dan Herschlag, Department of Biochemistry, Stanford University, Stanford, CA. I am greatly indebted to Dr Herschlag for his support and for stimulating discussions. I also thank him, Geeta Narlikar and Angelo Merli for a critical reading of the manuscript; and Leonid Beigelman and Lara Maloney (Ribozyme Pharmaceuticals Inc., Boulder, CO) for synthesizing the RNA oligonucleotides used in this study.

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APPENDIX

Derivations of equations 3, 4 and 5

The equations are derived from the model shown in Figure 2C by applying the following approximations: (i) the association–dissociation steps involving the Mg$^{2+}$ and the H$^+$ ions are much faster than the chemical cleavage; (ii) the critical Mg$^{2+}$ ion is not significantly bound and the critical proton is not significantly dissociated in the ground state, so that the only ES species appreciably populated in the ground state is ES OH (note that this assumption may not be strictly valid, in particular at 100 mM Mg$^{2+}$, and must be considered only a useful simplification).

The rate of cleavage of the ribozyme-substrate complex can be defined as:

$$v_{\text{cleav}} = -\frac{d[\text{ES}^{\text{OH}}]}{dt} = k_{\text{chem}} \times [\text{ES}^{\text{OH}}]$$  \hspace{1cm} \text{A1}$$

and, according to equation 2 in the Results:

$$\ln(k_{\text{cleav}}) = -\frac{\Delta H^\ddagger_{\text{cleav}}}{RT} + \frac{\Delta S^\ddagger_{\text{cleav}}}{R} + \ln \left( \frac{k_B T}{h} \right)$$  \hspace{1cm} \text{A2}$$

where $\Delta H^\ddagger_{\text{cleav}}$ and $\Delta S^\ddagger_{\text{cleav}}$ are the apparent enthalpy of activation and entropy of activation, respectively, for the overall cleavage.

According to the model, the overall cleavage rate is proportional to the concentration of $\text{ES}^{\text{OH}}_{\text{Mg}^{2+}}$, which in turn depends on $[\text{H}^+]$, on $[\text{Mg}^{2+}]$ and on the thermodynamic constants for the association–dissociation processes:

$$v_{\text{cleav}} = k_{\text{chem}} \times [\text{ES}^{\text{OH}}_{\text{Mg}^{2+}}] = k_{\text{chem}} \times [\text{ES}^{\text{OH}}] \times K_{\text{bind}} \times K_{\text{diss}} \times [\text{Mg}^{2+}] / [\text{H}^+]$$  \hspace{1cm} \text{A3}$$

By equalling $A_1$ and $A_3$, one arrives at the following equation (identical to equation 3 in Fig. 2C):

$$k_{\text{cleav}} = k_{\text{chem}} \times K_{\text{bind}} \times K_{\text{diss}} \times [\text{Mg}^{2+}] / [\text{H}^+]$$  \hspace{1cm} \text{A4}$$

One can then take logarithms and treat $\ln(k_{\text{chem}})$ according to equation 2 in the Results and $\ln(K_{\text{bind}})$ and $\ln(K_{\text{diss}})$ according to the thermodynamic relationship:

$$\ln(K) = -\frac{\Delta H^\ddagger_{\text{chem}}}{RT} + \frac{\Delta S^\ddagger_{\text{chem}}}{R} + \ln \left( \frac{[\text{Mg}^{2+}]}{[\text{H}^+]} \right)$$  \hspace{1cm} \text{A5}$$

It follows that:

$$\ln(k_{\text{cleav}}) = -\frac{\Delta H^\ddagger_{\text{chem}} + \Delta H^\ddagger_{\text{bind}} + \Delta H^\ddagger_{\text{diss}}}{RT} + \frac{\Delta S^\ddagger_{\text{chem}} + \Delta S^\ddagger_{\text{bind}} + \Delta S^\ddagger_{\text{diss}}}{R} + \ln \left( \frac{[\text{Mg}^{2+}]}{[\text{H}^+]} \right) + \ln \left( \frac{k_B T}{h} \right)$$  \hspace{1cm} \text{A6}$$
By comparing equations A2 and A6, one can draw the following relationships (corresponding to equations 4 and 5 in Fig. 2C):

$$\Delta H_{\text{cleav}}^f = \Delta H_{\text{chem}}^f + \Delta H_{\text{Mgbind}}^0 + \Delta H_{\text{lid}}^0$$  \hspace{1cm} A7

$$\Delta S_{\text{cleav}}^f = \Delta S_{\text{chem}}^f + \Delta S_{\text{Mgbind}}^0 + \Delta S_{\text{lid}}^0 + R(\ln[Mg^{2+}] - \ln[H^+])$$  \hspace{1cm} A8

**Derivation of equations 6 and 7**

The model in Scheme 1 assumes that ES\text{OH} can rapidly and reversibly convert to an unproductive conformation (ES\text{u}OH). The total concentration of ground state is:

$$[ES]_{\text{Total}} = [ES^{OH}] + [ES^{OH}] = [ES^{OH}] \times (1 + K_{\text{conf}})$$  \hspace{1cm} A9

The observed rate of cleavage is given as:

$$v_{\text{obs}} = k^{\text{obs}}_2 \times [ES]_{\text{Total}} = k^{\text{obs}}_2 \times [ES^{OH}] \times (1 + K_{\text{conf}})$$  \hspace{1cm} A10

But since cleavage can occur only from ES\text{OH} it follows that:

$$v_{\text{obs}} = k_{\text{cleav}} \times [ES^{OH}]$$  \hspace{1cm} A11

By substituting A10 into A11 and rearranging, one obtains the following relationship (equation 6 in the Results):

$$k^{\text{obs}}_2 = \frac{k_{\text{cleav}}}{1 + K_{\text{conf}}}$$  \hspace{1cm} A12

and, by taking logarithms and applying equations A2 and A5:

$$\ln(k^{\text{obs}}_2) = \ln(k_{\text{cleav}}) - \ln(1 + K_{\text{conf}})$$  \hspace{1cm} A13

$$\ln\left(\frac{k^{\text{obs}}_2}{RT} \right) = -\frac{\Delta H_{\text{cleav}}^f}{RT} + \frac{\Delta S_{\text{cleav}}^f}{R} \ln\left(\frac{k_B T}{h}\right) - \ln\left(1 + e^{\frac{-\Delta G_{\text{conf}}^0}{RT} + \frac{\Delta S_{\text{conf}}^0}{R}}\right)$$  \hspace{1cm} A14

Equation A14 corresponds to equation 7 in the Results.