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Cryoenzymology of the hammerhead ribozyme

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ABSTRACT

The technique of cryoenzymology has been applied to the hammerhead ribozyme in an attempt to uncover a structural rearrangement step prior to cleavage. Several cryosolvents were tested and 40% (v/v) methanol in water was found to perturb the system only minimally. This solvent allowed the measurement of ribozyme activity between 30 and −33 °C. Eyring plots are linear down to −27 °C, but a drastic reduction in activity occurs below this temperature. However, even at extremely low temperatures, the rate is still quite pH dependent, suggesting that the chemical step rather than a structural rearrangement is still rate-limiting. The nonlinearity of the Eyring plot may be the result of a transition to a cold-denatured state or a glassed state.

Keywords: conformational change; kinetics; temperature dependence

INTRODUCTION

Cryoenzymology is a tool for studying enzyme reaction mechanisms at low temperatures that has been used extensively on proteins (Makinen & Fink, 1977; Fink & Geeves, 1979; Douzou, 1983; Travers & Barman, 1995). The technique involves adding a cryoprotectant, often an organic solvent such as methanol, ethylene glycol, or DMSO, to prevent the enzyme solution from freezing and thereby allowing kinetic analysis at low temperature. By extending the temperature range of the kinetic studies to well below 0 °C, intermediates along a reaction pathway may be observed that are otherwise undetectable. When these intermediates have spectroscopic features, they can be observed directly, but their presence can also be inferred by changes in the kinetic behavior of the system. For example, a change in the rate-determining step of the reaction at some low temperature might be observed. This phenomenon often results in curved Eyring or Arrhenius plots, which derive from the intersection of the two lines, each representing a different elementary process of a composite rate constant (Travers & Barman, 1995).

The hammerhead ribozyme is a small RNA that catalyzes a self-cleavage reaction (Fig. 1) (McKay, 1996; Thomson et al., 1996; Zhou & Taira, 1998). In vitro studies of this ribozyme generally employ two RNA fragments such that one can be considered the ribozyme and the other the substrate. The standard kinetic scheme for these hammerheads involves substrate binding to form the enzyme–substrate complex (ES), cleavage of the phosphodiester bond, and product release (Fedor & Uhlenbeck, 1992; Hertel et al., 1994). Whereas this scheme adequately describes the overall reaction, several laboratories have proposed that at least one additional, albeit kinetically unobserved, step must occur after substrate binding and prior to cleavage to form an active intermediate, ES′ (Fig. 1B). Two reasons prompted these suggestions. First, two independent X-ray crystal structures (Pley et al., 1994; Scott et al., 1995, 1996) showed that the conformation around the cleavage site was inappropriate for in-line attack by the 2′-OH group on the adjacent phosphodiester bond. Second, a good deal of biochemical data was inconsistent with the crystal structure being close to an active conformation. For example, substitution of the functional groups of G5 in domain I (Ruffner et al., 1990; Tuschl et al., 1993) and certain 2′-OH (Williams et al., 1992) and phosphate oxygens in domain II (Ruffner & Uhlenbeck, 1990; Peracchi et al., 1997) completely abolish activity, but do not interact with other residues in the crystal structure. Because preliminary NMR data suggest that the crystal structure closely reflects the major solution conformation (Heus & Pardi, 1991; Simorre et al., 1997, 1998), it appears that the structure must change significantly in order to reach the transition state. Whereas it is unclear how different the structure of ES′ is from ES, it would be expected to explain more accurately the mutagenesis data.

Proposing an intermediate such as ES′ has several consequences on the way we must think about the kinetics of hammerhead ribozyme cleavage. The most

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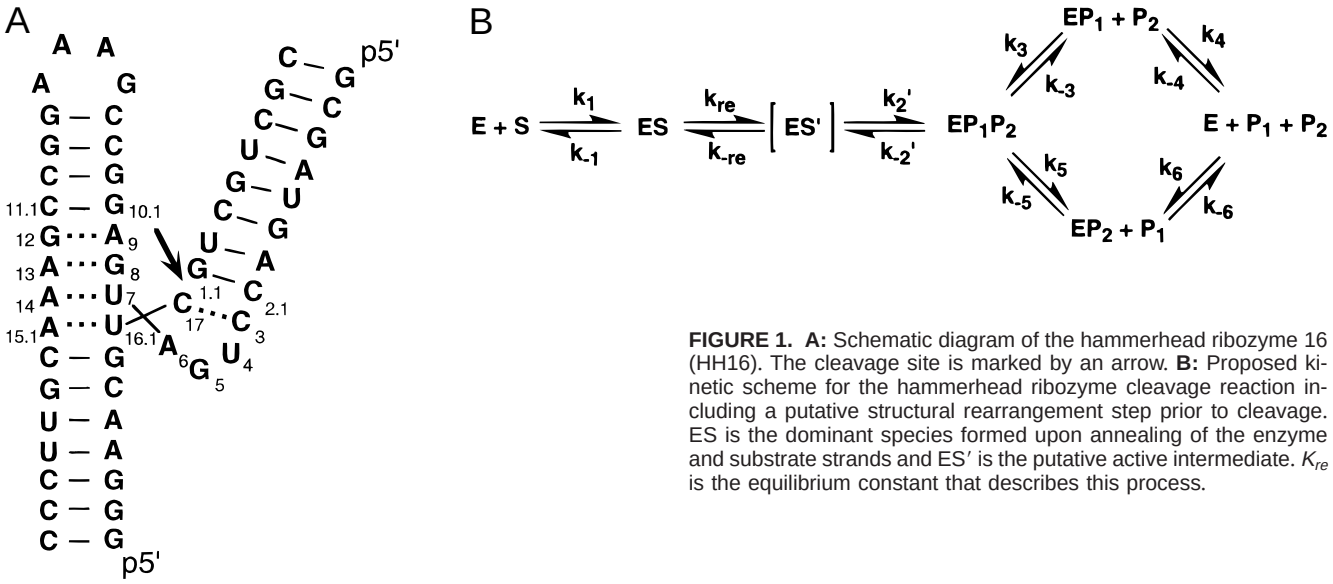

FIGURE 1. A: Schematic diagram of the hammerhead ribozyme 16 (HH16). The cleavage site is marked by an arrow. **B:** Proposed kinetic scheme for the hammerhead ribozyme cleavage reaction including a putative structural rearrangement step prior to cleavage. ES is the dominant species formed upon annealing of the enzyme and substrate strands and ES′ is the putative active intermediate. K_{re} is the equilibrium constant that describes this process.

important issue is that the rate constant from single-turnover experiments, previously called k_2, now must be considered a composite value $K_{re} \cdot k_2'$ where $K_{re} = k_{re}/k_{-re}$. At 25 °C, both k_{-re} and k_{re} must be quite fast compared to k_2' in order for this pre-equilibrium to be unobserved kinetically. If these rates can be reduced sufficiently, they would be observed and measured much more easily. It is also reasonable to expect that K_{re} is quite small ($\leq$0.01) to account for the fact that the major conformation in solution is ES and not ES′. The experiments reported here are designed to search for conditions where the formation of the putative intermediate along the hammerhead ribozyme reaction path is rate-limiting ($k_{re} < k_2'$). In these experiments, high pH has been used to increase the rate of the chemical step, k_2', as much as possible without denaturing the ribozyme. At the same time, the temperature has been lowered to slow k_{re}. Although the low temperature will slow down k_2' as well, it is hoped that the temperature dependence of the rearrangement is even greater, such that k_{re} becomes rate-limiting at some low temperature. Before this type of work can be performed, however, suitable cryosolvent systems must be identified and it must be shown that the co-solvent does not adversely affect the mechanism of the reaction.

RESULTS

Search for a suitable cryosolvent

The activity of the hammerhead ribozyme was determined in mixtures of aqueous buffer and organic co-solvents at room temperature and at 10 °C (Fig. 2; Table 1). In most cases, low concentrations of co-solvents had little or no effect on the cleavage rate. At higher concentrations, however, a sharp decline in activity was observed over a very narrow range of additional co-solvent. The point at which the co-solvent affected activity varied from solvent to solvent. For instance, in methanol, no effect on the rate was observed until 40% methanol was added. By 60% MeOH, activity was only 1% of that in pure water. Concomitant with reduced rate, the terminal extent of cleavage also

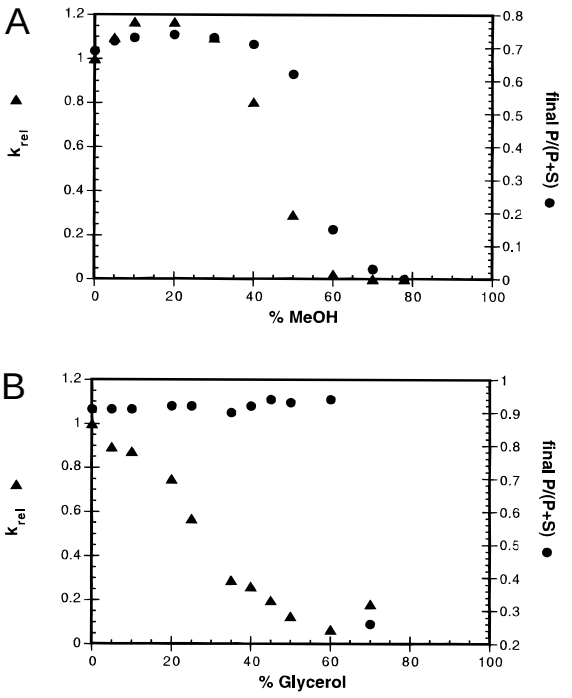

FIGURE 2. Effect of methanol (**A**) and glycerol (**B**) on the rate (▲) and terminal extent of cleavage (●) for the hammerhead ribozyme at 25 °C.

TABLE 1. χ_{max} for a variety of potential cryosolvents for use with the hammerhead ribozyme.

Co-solvent	χ_{max}[a] (% v:v)	Freezing point[b] (°C)
CH_3CN	>50	−20[c]
CH_3OH	40	−42[c]
C_2H_5OH	30	−38[c]
DMF	30	−13
DMSO	30	−20
Ethylene glycol	30	−17
Glycerol	20	−10

[a] χ_{max} = vol_{MeOH}:vol_{total} at which the hammerhead ribozyme still showed at least 80% of the activity in water under similar conditions.
[b] Data taken from (Douzou et al., 1976).
[c] Measured independently.

declined. Glycerol/water mixtures were the exception. Under these conditions, the cleavage rate dropped roughly linearly with the amount of added co-solvent, but the reaction continued to completion up until very high (~60%) concentrations. An acceptable amount of co-solvent was arbitrarily chosen to be the point at which 80% of the activity in aqueous solution was retained. Table 1 lists the maximal concentrations of the co-solvents tested with the corresponding freezing points of the solvent mixtures. When measured at multiple temperatures, χ_{max} values were invariant. The methanol–water mixture provides the greatest available temperature range for cryoenzymological experiments on the hammerhead ribozyme.

There are two issues that must be considered relating to experiments in mixed solvent systems. The first pertains to the possibility that either the addition of the organic solvent or the large temperature change significantly alters the solution volume. This issue is important because "standard" hammerhead ribozyme conditions, such as those used here, are subsaturating with respect to Mg(II). Volume changes may therefore affect the observed reaction rates by changing its concentration. Fortunately, these volume effects are quite small. For a 40% methanol/water solution, we measured a volume loss of ~2% following isothermal mixing and about a 5% additional contraction as the temperature is reduced from 25 to −30 °C. The resulting ~7% increase in the concentrations of Mg(II), ribozyme, and buffer in solution will have a negligible effect on the reaction rate.

The second issue that must be considered is the effect of the change in water concentration and H^+ activity on pH. In pure aqueous solution, the reaction has a first-order dependence on [OH^-] between pH 5 and 9 (Clouet-d'Orval & Uhlenbeck, 1996; Hertel et al., 1996). In order to account for the changes caused by the mixed-solvent system, a value called effective pH or pH* is defined to replace pH (Fink & Geeves, 1979). The pH of the sulfonate buffers in water was found to be 0.1 pH units higher than the pH* in 40% (v/v) methanol/water (data not shown). Like the volume corrections discussed above, this shift results in a relatively minor perturbation of the rates when comparing the reactivity of a ribozyme in water to that in 40% methanol. Considering the known pH dependence of the cleavage reaction in water, the pH* dependence of the rate can therefore be viewed as a diagnostic indicator for proper enzymatic behavior. The cleavage rate was therefore measured as a function of pH* at 10 °C. The absolute rates as well as the pH dependence was found to be similar to that observed in 100% aqueous solution (Fig. 3).

Low-temperature experiments

Kinetic experiments were performed under single-turnover conditions between 30 and −33 °C. Because hammerhead ribozyme structural rearrangements are generally metal ion-dependent (Bassi et al., 1995, 1996, 1997), reactions were initiated by addition of Mg(II) to the annealed ES complex. Progress curves were taken to completion between 30 and −15 °C. However, for temperatures below −15, the slow rates made it impractical to follow the reactions to completion during every time course, so initial rates were used, assuming the same extent of completion at infinite time. The terminal extent of cleavage was monitored under a variety of conditions, however. Data were corrected for the temperature dependence of the buffer pK_a, assuming a first-order dependence on [OH^-] (see below). Furthermore, because the reaction is reversible, the observed kinetic constant is the sum of the forward and backward reaction rates. These rates have been deconvoluted as described in Materials and Methods. An Eyring analysis (Fig. 4) of the cleavage rate data between 30 and −27 °C yields $\Delta H^{\ddagger} = 22 \pm 1$ kcal mol^{-1} and $\Delta S^{\ddagger} = 4 \pm 4$ cal mol^{-1} K^{-1}. These values are in excellent agreement with those measured previously in

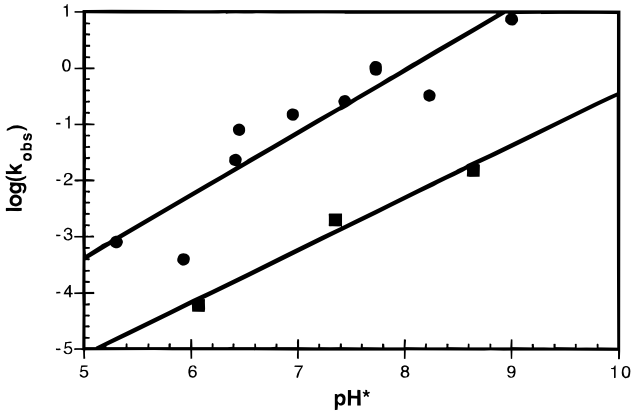

FIGURE 3. Dependence of k_{obs} on pH* for HH16 at 10 °C 40% (v/v) $MeOH/H_2O$ (●) and at −25 °C in 40% (v/v) $MeOH/H_2O$ (■).

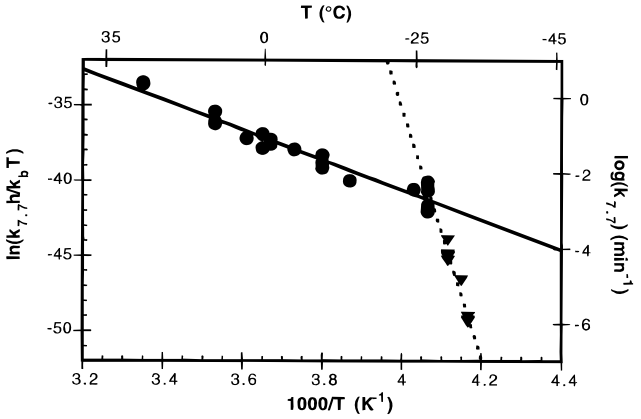

FIGURE 4. Eyring plot for the cleavage of HH16 in 40% (v/v) MeOH/H_2O between 25 and −33 °C. Data have been corrected to pH* = 7.7 as discussed in Materials and Methods to account for the change in pH* as a function of temperature. k_b is Boltzman's constant, T is the temperature in Kelvin, and h is Planck's constant. Values on the top and right axes are provided for reference only. The solid line is a linear least-squares regression to the data between 30 and −27 °C and the dotted line is a regression of the data below −27 °C.

aqueous solution for three different hammerheads under more limited temperature ranges (Table 2) (Hertel & Uhlenbeck, 1995; Takagi & Taira, 1995; Clouet-d'Orval & Uhlenbeck, 1996).

Below −27 °C, the kinetic behavior of hammerhead 16 changes significantly and can no longer be fit by extrapolating data from the higher temperature region. Instead, the cleavage rate decreases rapidly with decreasing temperature. Whereas there is significant error (~50% based on replicate measurements) in determining these very slow reaction rates, the error is much smaller than the deviation from the predicted rates (Fig. 4). These low-temperature data were collected well above the freezing point of the solvent mixture and therefore the kinetic constants should not be affected by this transition. The low-temperature region appears to have its own linear temperature dependence, but only an extremely narrow range of temperatures (−29 to −33 °C) was accessible, making the determination of activation parameters error prone.

Two experiments were performed in an attempt to determine if a new rate-limiting step was operative at these very low temperatures. The first was to examine the pH* dependence of the reaction. If a new rate-limiting step involves a conformational change, the log-linear pH*-rate profile associated with the chemical step (k_2') might no longer be observed. At −25, the pH* dependence showed a first-order dependence on [OH^-] similar to that observed at 10 °C (Fig. 3). At −30 °C, however, anomalous behavior was observed. When the rate was re-examined at pH* = 8.5, which is one unit lower than the data shown in Fig. 4, the cleavage occurred at $(4 \pm 2) \times 10^{-6}$ min^{-1}, 50–100-fold slower than the rate at pH* 9.5. Although the very slow rate made an accurate determination of the cleavage activity difficult, it was clear that the pH* dependence remained.

A second experiment attempting to analyze the potential new rate-limiting step involved altering the reaction protocol. If a metal-dependent conformational isomerization had become rate-limiting at low temperature, it is possible that changing the initiation protocol would result in a different observed cleavage rate. Therefore, we preincubated the ES complex at low pH* in the presence of Mg(II) prior to equilibration to the reaction temperature. The reactions were then initiated by increasing the pH*. By allowing the ES ⇌ ES′ equilibrium to be reached at low pH*, one might observe a burst of cleavage upon raising the pH* of the solution.

TABLE 2. Activation parameters measured for the cleavage reaction of the hammerhead ribozyme.

	k_2		k_{-2}			
Construct	$\Delta H^‡$ (kcal/mol)	$\Delta S^‡$ (cal/mol K)	$\Delta H^‡$ (kcal/mol)	$\Delta S^‡$ (cal/mol K)	Temp. range (°C)	Ref.
HH16	22 ± 1	4 ± 4[a]	13 ± 1	−32 ± 4[a]	30–−25	This work
HH16	21 ± 1[b]	3 ± 0.2[b]	10 ± 2[b]	−43 ± 10[b]	35–1	[c]
HHα1	21 ± 1[d]	n.d.	n.d.	n.d.	25–1	[e]
R32/S11	15.4[f]	−14.6[f]	n.d.	n.d.	25–45	[g]

[a]Calculated from a linear least-squares regression of data plotted as $T \cdot \ln(k/T)$ versus T, the slope of which is equal to $\Delta S^‡/R$. This plot minimizes the statistical correlation between the activation enthalpy and the activation entropy (Makinen et al., 1979). Error values were propagated from the least squares fitting parameters.

[b]Value calculated at 298 K from an Arrhenius plot rather than through the use of an Eyring plot.

[c]Data taken from (Hertel & Uhlenbeck, 1995).

[d]Activation energy was reported rather than $\Delta H^‡$.

[e]Data taken from (Clouet-d'Orval & Uhlenbeck, 1996).

[f]Values were calculated at 308 K from an Arrhenius plot rather than through the use of an Eyring plot. This construct undergoes a change in the rate-determining step at 298 K. Product release becomes limiting below this temperature. Therefore, a limited range of temperatures was available for this study. No error analysis was provided, but it may be significant due to the narrow temperature window and limited number of data points.

[g]Data taken from Takagi and Taira (1995).

This experiment was performed as an order of addition experiment to ensure that the reactions came to the same final pH* regardless of whether Mg(II) or pH* 8.5 buffer was added first. However, when this experiment was performed at both 25 °C and −30 °C, no difference in the kinetic behavior was observed.

DISCUSSION

In these cryoenzymological studies, we have found that the hammerhead ribozyme behaves very much like protein enzymes. As organic solvents are added, HH16 cleaves normally until abrupt loss of activity occurs over a narrow range of concentrations of co-solvent, suggesting that a denaturation transition might have occurred. Whereas the transition occurs at relatively high concentrations of most co-solvents, it occurs at slightly lower concentrations than observed for most protein systems (Fink, 1976). The accessible temperature range in 40% methanol allows kinetic measurements to about −40 °C, which is a significant improvement over the 0 °C freezing point of water. In most cases, the decrease in the extent of cleavage parallels the rate loss. There is no simple mechanism to explain this correlation, but it may indicate a process in which a subpopulation is trapped in an inactive conformation. This inactivation could result from either denaturation, the loss of a critical structural feature, or the formation of additional structures that are kinetically unfavorable. We have not explicitly ruled out the formation of small aggregates at low temperature under these conditions. Had the RNA precipitated, however, a significant loss of radioactivity would have been expected prior to electrophoresis. Under certain conditions [high co-solvent and high Mg(II)], this effect was indeed observed. Other possibilities for this rate loss include: changes in the behavior of the metal ion cofactor due to the alteration of its coordination environment, or the formation of off-pathway complexes.

Glycerol deviated significantly from the other co-solvents tested. The reaction rate decreased well before an effect was observed on the extent of reaction. It is possible that the glycerol acted as an inhibitor of hammerhead ribozyme cleavage, either by binding weakly to a site on the RNA or by chelating Mg(II). Alternatively, the high viscosity of glycerol/water solutions may be responsible for the observation. Although glycerol clearly is not useful as a cryoprotectant for the hammerhead ribozyme, its effect on hammerhead ribozyme catalysis is interesting and deserves further study.

The denaturation of tRNAs (Schweizer, 1969; Crawford et al., 1971; Prinz et al., 1974) and short RNA duplexes (Breslauer et al., 1978) by organic solvents has been studied previously in great detail. It was found that co-solvents have a relatively predictable effect such that increasing their concentrations lowers the T_m of the RNA. In these studies, short aliphatic alcohols such as methanol and ethanol were the least disruptive, causing a change in the T_m of only 3 °C per 10% for methanol and 8 °C per 10% for ethanol. It is therefore unlikely that the denaturation seen at 40% methanol is due to disruption of the Watson–Crick base pairing of the ES complex. The arms of HH16 are quite long and the T_m has been measured to be approximately 65 °C in 10 mM $MgCl_2$ (A.L. Feig & O.C. Uhlenbeck, unpubl. data). Therefore, if the molecule is being denatured above 40% MeOH at 25 °C, it must result from the disruption of one or more tertiary structural elements required for activity. Destabilization of either the uridine turn in domain I or the non-Watson–Crick interactions in domain II could lead to this activity loss.

In two cases, addition of an organic co-solvent has been reported to stimulate ribozyme catalysis (Gardiner et al., 1985; Hanna & Szostak, 1994). In one case, *Bacillus subtilis* RNase P was analyzed under low-salt conditions. The change of the dielectric constant of the solvent due to the added ethanol was proposed to be responsible for the observed effect. In the other case, addition of ethanol suppressed unfavorable mutations to the *Tetrahymena* group I ribozyme. In our case, there is a slight ($k_{re} \sim 1.2$) increase in activity at 10 and 20% (v/v) MeOH. This increase might also be due to the dielectric effects of the solvent because these studies were performed under subsaturating conditions with respect to Mg(II).

The temperature dependence of the cleavage rate did not change down to about −25 °C. The activation parameters measured over this 55° temperature range were identical to the ones measured previously in 100% aqueous solutions. An interesting feature in the Eyring plot occurs below −27 °C, where anomalously slow cleavage occurs, indicating that a new process becomes rate-limiting. There are at least three possible interpretations for this low-temperature process: (1) a conformational change on the cleavage pathway is rate-limiting; (2) the RNA is undergoing a phase transition (glassing) where catalytic activity is hampered by the disruption of its normal dynamic processes; and (3) a cold denaturation transition of the hammerhead ribozyme structure occurs.

A change in rate-limiting step of a complex reaction is the classic example from which a curved Eyring plot derives. Although we set out to find such conditions, it is unlikely that this interpretation is correct because the reaction is at least as pH* dependent at −30 °C as it is at higher temperatures. Because a pH* dependence of the cleavage rate is generally taken to indicate that the cleavage step is rate-limiting, the only way to justify a rate-limiting conformational change is to assume that the structural rearrangement is also sensitive to pH*. In RNA, such rearrangements are generally not affected by pH because the nucleotides do not titrate in this range. Although examples of titratable groups in the

neutral pH range involving certain noncanonical interactions have been reported (Legault & Pardi, 1997; Limmer, 1997), there is no evidence for one of these special cases in the hammerhead system.

The second possible explanation for the curvature is that the hammerhead ribozyme undergoes a freezing or glassing transition. In protein systems, the symptom of this transition is that substrate or inhibitor binding ceases to occur at some low temperature (Rasmussen et al., 1992). Below this critical temperature, the motions become too slow to prevent rapid release of the substrate following the collision; therefore, binding is not possible. There is a relatively narrow temperature range (−60 to −70 °C) in which this transition has been proposed to occur in proteins (More et al., 1995). It is not known at what temperature such a process could occur in RNA molecules. The temperature at which the deviation in the kinetic behavior occurs (−27 °C) is much higher than that observed for the glassing of proteins. Although it is possible that the internal motions of an RNA are frozen at this higher temperature, it is, in our opinion, not very likely.

Cold denaturation is quite well documented in protein systems (Creighton, 1993). It occurs because of a combination of two factors: (1) when ΔG_{fold} has a significant entropic term, it becomes less influential at low temperatures, and (2) protein unfolding tends to have a large ΔC_p. For proteins, the temperatures at which this unfolding becomes important are widely varied. One example of cold denaturation of an RNA has been reported, that being P1 docking in the *Tetrahymena* Group I intron, which undocks at low temperature (Narlikar & Herschlag, 1996). Here, the behavior was very similar to that observed for proteins. The main difference between the RNA and protein systems was the significant ionic contribution to the phase diagram, a feature that makes sense in light of the requirement of metal ions to properly fold large RNAs.

Unfortunately, it has proven particularly difficult to perform more extensive kinetic studies of the hammerhead ribozyme in this cryosolvent at −30 °C. The main problem is the extremely slow reaction rates. Even at pH* 9.6, the rates are on the order of 10^{-4} min^{-1}, corresponding to a half-life of 4.8 days. Based on the apparent temperature dependence in this region, one expects a 10-fold rate loss approximately every 2°. Although the solvent will not freeze until below −40 °C, the cleavage rate becomes vanishingly small very quickly. In fact, these rates will be significantly slower than the decay of the ^{32}P used to visualize the RNA. It would be hard to increase the pH* further because the bases would protonate and divalent ions would become even less soluble. Higher concentrations of Mg(II) or other divalent ions are also not an option because of their low solubility under these conditions. Therefore, to further probe the cleavage kinetics at low temperature, we would have to use other cryosolvents or intrinsically faster hammerheads (Burgin et al., 1996; Clouet-d'Orval & Uhlenbeck, 1997).

Even though the temperature window in which this new kinetic regime can be observed is very narrow, we have learned some useful things about it. First, the data indicate that the reaction is still dependent on the OH^- concentration, even at the lowest measured temperatures. Because we do not expect the rearrangement to have a pH* dependence, we infer that the chemical step is not kinetically silent under these conditions. Second, the extreme temperature dependence indicates that the low temperature process is driven by the very large favorable entropic term and might pertain to a cold denaturation transition.

The relatively minor effects of cryoprotectants on the hammerhead cleavage reaction now allows for a wide variety of experimental approaches to probe the rapid structural equilibria that might be important for RNA catalysis. These kinetic studies provide loose guidelines with respect to the types and concentrations of co-solvents that might be useful for other RNA enzymes. This methodology can also be applied to noncatalytic RNAs so long as a spectroscopic handle such as a fluorophore can be incorporated into the RNA to report on the local structural changes that might be occurring. We have observed a kinetic regime at low temperature that deviates from the normal, room-temperature, behavior. We cannot yet say conclusively what the nature of the rate-limiting step is under these conditions. It may be the proposed structural rearrangement, but there are also several other possibilities that must be explored. The next phase of this project will use spectroscopic tools to probe the hammerhead ribozyme under these conditions.

MATERIALS AND METHODS

Preparation of RNAs

The hammerhead construct HH16 (Hertel et al., 1994) was used in these experiments. The substrate strand was synthesized by standard automated phosphoramidite methods (Eckstein, 1991). Deprotected oligonucleotides were gel purified prior to use. The ribozyme strand was prepared by in vitro transcription with T7 RNA polymerase off of a Milligan template (Milligan et al., 1987). RNA concentrations were determined based upon the A_{260} and using the approximation of $\epsilon \sim 10{,}000\ M^{-1}\ cm^{-1}\ nt^{-1}$. 5′-[$^{32}P$]-labeled substrates were prepared in T4 polynucleotide kinase reactions containing cold RNA (10–20 pmol), γ-[^{32}P]-ATP, PNK (500 U/mL) (NEB), and 1×PNK buffer (NEB), incubated at 37 °C for 30–60 min.

Kinetic analysis

Cleavage reactions (20 μL final volume) were performed under single-turnover conditions in the presence of 5 μM HH16 ribozyme, trace 5′-[^{32}P]-labeled HH16 substrate, 10 mM

$MgCl_2$, and 50 mM buffer (MES for pH 5.2–6.64, PIPES for pH 6.45–7.73, TAPS for pH 7.75–9.25, and CHES for pH 9.43–10.43). Ribozyme and substrate oligos were annealed in the absence of $MgCl_2$ at 95 °C for 90 s and cooled to room temperature. Condensation that collected on the walls of the tube was spun down to the bottom by brief centrifugation. After equilibrating the reactions to the desired temperature, they were initiated by adding 2 μL of cold 100 mM $MgCl_2$ in the appropriate cryosolvent to the 20 μL reactions. The reactions were quenched by a 1:1 dilution into a stop buffer containing 100 mM EDTA, 7 M urea, and 0.1% each of bromophenol blue, xylene cyanol, and orange G in TBE buffer. Samples were run out on 20% denaturing polyacrylamide gels and exposed to phosphorimager plates. Data were quantitated by using a Molecular Dynamics phosphorimager running ImageQuant 3.0 software (Molecular Dynamics, Sunnyvale, California). Rate constants were determined by using the Kaleidagraph software, v. 3.0 (Synergy Software, Reading, Pennsylvania), plotting the fraction of product $[(P)/(P + S)]$ versus time and fitting by nonlinear least-squares regression to standard first-order kinetic equations (Stage-Zimmermann & Uhlenbeck, 1998). For very slow reactions, initial rates were determined based upon a linear fit of the same data. Temperature control between 4 and −33 °C was maintained by using a Lauda model RM6 circulating bath filled with 90% (v/v) methanol/water solution. Between 37 and 4 °C, a Lauda RTE-210 constant temperature bath was used.

Correction factors

Values for pH were converted to pH*, the effective pH in the nonaqueous medium. The offset between water and 40% methanol solutions was measured by using a standard glass electrode pH meter equilibrated against the 40% methanol solution as described elsewhere (Fink & Geeves, 1979). Under our solvent conditions, the correction is −0.1 pH units (data not shown). Temperature-dependent pH* changes have also been taken into consideration. Values for the change in pK_a of the buffer with respect to temperature are: −0.0085 ΔpH/°C for PIPES, −0.018 for CHES, −0.011 for MES, and −0.020 for TAPS (Sober, 1970). Rate constants were adjusted to a constant pH* value by using Equation 1:

$$k_{pH^*} = k_{obs} * 10^{(pH^* - pH^*_T)} \quad (1)$$

in which k_{pH^*} is the adjusted rate constant, k_{obs} is the experimentally determined rate constant, pH^* is the effective pH to which the rate data have been normalized and pH^*_T is the actual pH* at a given temperature after correcting for the pK_a of the buffer. Volumes of mixing and the temperature-dependent solvent contractions were measured for 40% MeOH solutions by using glass graduated cylinders immersed in cooling baths. Due to the insignificance of the volume effects, the data have not been corrected for either contraction. The actual concentrations of buffer, magnesium, and ribozyme are a few percent greater than reported. Because the experiments are run under subsaturating conditions of Mg(II), the small increase in the Mg(II) concentrations will accelerate the reactions slightly, but this effect is smaller than the reproducibility error in the data.

Because the reaction is reversible, k_{pH^*} is the sum of the forward and reverse rate constants (Equation 2). At high temperatures, $k_{pH^*} \sim k^{for}_{pH}$, but this approximation loses validity at sufficiently low temperatures. By using the thermodynamic parameters ΔH° and ΔS° for the internal equilibrium, which have been measured previously for HH16 to be 9 kcal/mol and 36 cal/mol K, respectively (Hertel & Uhlenbeck, 1995), the values for the forward and reverse reactions can be calculated (Equations 2 and 3). These values for the reaction rates were then used in the Eyring plot to determine the activation parameters for each reaction.

$$k_{pH^*} = k^{for}_{pH^*} + k^{back}_{pH^*} \quad (2)$$

$$k^{back}_{pH^*} = \frac{k_{pH^*}}{1 + K_{\text{int}}} \quad (3)$$

pH* Jump experiments

Ribozyme 16 (5 μM) and 5′-[^{32}P]-substrate strand (trace) were annealed at 90 °C for 90 s in 10 mM acetate buffer, pH* 5.0, and 40% MeOH. This solution was allowed to cool to room temperature. To half of this solution (60 μL), 20 μL 250 mM TAPS buffer in 40% MeOH, pH* = 8.5 was added. To the other 60 μL, 20 μL 50 mM $MgCl_2$ in 40% MeOH was added. After equilibrating for 15 min, these solutions were cooled to the reaction temperature (−30 or 25 °C) and allowed to equilibrate for an additional 60 min. The high pH* solution was initiated by addition of cold $MgCl_2$ solution and the Mg(II) containing reaction was initiated by addition of cold TAPS buffer. Time points were quenched and analyzed as described above for the standard kinetic reactions.

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