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A comparison of the *in vitro* activity of DNA-armed and all-RNA hammerhead ribozymes

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ABSTRACT

Hammerhead ribozymes targeted against two unrelated RNA substrates have been prepared. For each substrate, four ribozymes, differing in their hybridising arm length and composition (DNA or RNA), have been synthesised and kinetically characterised. The presence of DNA in the hybridising arms had little effect on the overall cleavage rate when the cleavage step was rate determining. Shortening each of the hybridising arms of ribozymes from 10 to 6 nucleotides generally resulted in modest changes in rate constants for cleavage of the same 13mer substrate. In one case the presence of long RNA hybridising arms significantly impeded the cleavage reaction. Cleavage rates displayed first order dependence on hydroxide ion concentration at low pHs. At higher pH, some ribozymes deviated from this first order dependence because of a change in the rate-determining step, possibly due to a requirement for a conformation change in the ribozyme–substrate complex prior to cleavage. Ribozyme cleavage was strongly dependent on temperature in the range 5–45°C, with an activation energy for the reaction of ~60 kJ mol^{-1}. The ribozymes displayed biphasic dependence on magnesium ion concentration; evidence of strong apparent binding (K_d ~10 mM) as well as a looser interaction was observed for all ribozymes.

INTRODUCTION

RNA cleavage at physiological pHs proceeds via attack of nucleophile at the phosphorus centre to produce a 5-coordinate phosphorane intermediate or transition state (1) which decomposes to yield the reaction products. Cleavage of RNA by the hammerhead ribozyme proceeds by a mechanism in which the nucleophile is a deprotonated 2′ hydroxyl group and the immediate reaction products terminate with a 5′ hydroxyl and a 2′–3′ cyclic phosphate. The reaction proceeds in a single step as demonstrated by inversion of configuration at the phosphorus centre of the cyclic product relative to the starting diester (2,3).

Since its description in 1986 (4–6), the hammerhead ribozyme has been the subject of intense investigation because of its potential use as a catalytic reagent for the sequence-specific cleavage of unwanted or deleterious RNA, e.g. viral or oncogene derived RNA (7–10). Hammerhead ribozymes have been shown to be effective in cultured cells either when expressed intracellularly (8,9) or when supplied exogenously (10,11). However, a major limitation to the efficacy of exogenously supplied hammerhead ribozymes *in vivo* is the instability of the RNA. One approach to overcome this has been to minimise the RNA content of ribozymes and to stabilise the remaining RNA with other modifications. As a first step in this strategy, we have made a number of DNA/RNA chimeric ribozymes. Several previous studies have appeared to show that the effect of replacing RNA in the hybridising arms of the ribozyme with DNA is to increase the rate of cleavage of substrate and the overall rate of turnover (12–15). This communication compares in detail the reactivity of RNA versus DNA armed ribozymes for two different short RNA substrates.

MATERIALS AND METHODS

Nomenclature

This study has investigated the cleavage kinetics of two unrelated RNA substrates by various hammerhead ribozymes. The sequences of the substrate molecules are taken from naturally occurring mRNAs and are identified by their origin. The first target sequence is from the Krüppel mRNA of *Drosophila melanogaster* and is identified by the prefix Kr. The second sequence is from the TAT gene of the ARV-2 isolate of HIV-1 (16), and is identified by the prefix TAT.

In this communication we use the term ribozyme to include all derivatives of the hammerhead ribozyme, despite the fact that they may contain other than ribonucleotides. Ribozymes are denoted by an R following the identifying prefix, and substrates by the letter S which are further identified by a number denoting their length in nucleotides, e.g., S13. There are two versions of hammerhead ribozyme used in this paper, and they are denoted as ribozymes A and B. Ribozymes A (RA) are composed solely of RNA (with the exception of the 3′ nucleotide) whereas ribozymes B (RB) possess DNA in the arms that hybridise to the substrate, with the exception of nucleotides 15.1 and 15.2 which remain as RNA (shown schematically in Fig. 1). The standard numbering system is used throughout (17).

Preparation of oligonucleotides

Oligonucleotides were synthesised using an Applied Biosystems (Foster City, CA) model 391 DNA synthesiser. Protected DNA phosphoramidite monomers were from Applied Biosystems or

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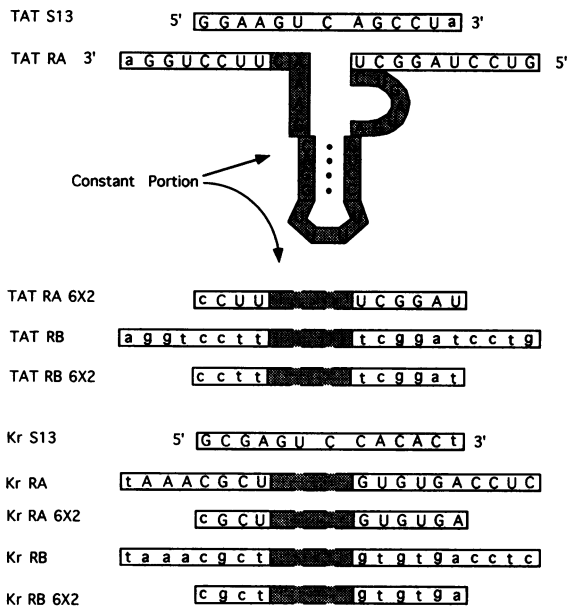


Figure 1. Schematic representation of DNA substitution patterns and sequences of substrates S and ribozymes RA and RB. Upper case letters represent ribonucleotides, lower case letters represent deoxyribonucleotides. Shaded portion is constant for all ribozymes.

Millipore (Bedford, MA). RNA monomers, protected at the 2′-hydroxyl with *tert*-butyldimethylsilyl groups, were from Millipore. For convenience in the syntheses, all the oligonucleotides have a deoxyribonucleotide at their 3′ end. Deprotection and purification of oligonucleotides was as described previously (18). The purity of each oligonucleotide was checked by labelling its 5′-end with [^{32}P]phosphate using T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA) and [γ-^{32}P]ATP (Bresatec, Adelaide, S.A., Australia), electrophoresing the molecules on a 10 or 15% polyacrylamide gel containing 7 M urea, and visualising the molecules by autoradiography or using a Molecular Dynamics PhosphorImaging system (Sunnyvale, CA); all oligonucleotides were at least 98% pure as judged by this assay. The concentrations of the purified oligonucleotides were determined by UV spectroscopy using the following molar extinction coefficients for the various nucleotides at 260 nm: A, 15.4×10^3; G, 11.7×10^3; C, 7.3×10^3; T/U, 8.8×10^3 l.mol^{-1}.cm^{-1}. All oligonucleotides were stored in distilled, deionised and autoclaved water at –20°C.

Oligonucleotide sequences

The oligonucleotides used in this study are as follows. Capital letters refer to ribonucleotides, lower-case letters refer to deoxyribonucleotides.

Krüppel System (Kr). Kr RA, 5′ CUCCAGUGUG CUGAUGA GUCC UUUU GGAC GAAAC UCGCAAAt 3′; Kr RA 6X2, 5′ AGUGUG CUGAUGA GUCC UUUU GGAC GAAAC UCGc 3′; Kr RB, 5′ ctccagtgtg CUGAUGA GUCC UUUU GGAC GAAAC tcgcaaat 3′; Kr RB 6X2, 5′ agtgtg CUGAUGA GUCC UUUU GGAC GAAAC tcgc 3′; Kr S13, 5′ GCGAGUCCACACt 3′.
TAT System (TAT). TAT RA, 5′ GUCCUAGGCU CUGAUGA GUCC UUUU GGAC GAAAC UUCCUGGa 3′; TAT RA 6X2, 5′ UAGGCU CUGAUGA GUCC UUUU GGAC GAAAC UUCc 3′; TAT RB, 5′ gtcctaggct CUGAUGA GUCC UUUU GGAC GAAAC ttcctgga 3′; TAT RB 6X2, 5′ taggct CUGAUGA GUCC UUUU GGAC GAAAC ttcc 3′; TAT S13, 5′ GGAAGU-CAGCCUa 3′.

Kinetic experiments

Ribozyme excess experiments. Unless otherwise stated, the kinetic experiments were performed at 37°C with ribozyme and substrate (labelled at the 5′ end with [^{32}P]phosphate) in 10 mM $MgCl_2$ and 50 mM Tris–HCl, pH 8.0, using the following procedure. The substrate concentrations were in the range 1–15 μM (typically 2 μM) and the ribozyme concentration was at least 1.5 times that of the substrate (typically 3 μM). The ribozyme and substrate together in buffer were pre-treated by heating to 85°C for 2–3 min, centrifuging briefly, and then placing at the reaction temperature for a few minutes. The reaction was initiated by the addition of $MgCl_2$. Samples were removed at various time intervals and quenched by addition to 2 vol of gel loading buffer containing 80% formamide and 20 mM EDTA. The fraction of substrate cleaved in each sample was determined by separation of the 13mer substrate from the 7mer 5′-product in a 15% polyacrylamide gel containing 7 M urea, and quantifying the amounts of each using a Molecular Dynamics PhosphorImaging system and ImageQuant software. The kinetic parameters were obtained by fitting the data for percentage of product formed (P_t) at any given time (t) to the equation:

$$P_t = P_\infty - (\exp(-k_{obs}t)P_\Delta)$$

where P_t is the amount of product at time t, P_∞ is the amount of product at $t = \infty$, k_{obs} is the first-order rate constant for the reaction and P_Δ is the difference between the percentage of product at $t = \infty$ and $t = 0$. This is a conventional first-order kinetic equation from which k_{obs}, P_∞ and P_Δ are determined by least-squares fitting of the data. P_∞ was typically in the region of 0.80, i.e. ~80% of the substrate was cleaved at the end of the reaction. The observed rate constants, k_{obs}, presented in Table 1 are the mean ± SD of at least two independent experiments.

Turnover kinetics. The enzyme kinetic parameters of the ribozymes were determined with ribozyme at 10 nM and substrate concentrations ranging from 100 to 10 000 nM, depending on the K_m for the particular ribozyme. In these experiments the reactions were started by adding 13mer substrate to the reaction mix containing ribozyme, $MgCl_2$, and buffer at the specified temperature. Reactions were usually followed for <10% consumption of the substrate. Samples were removed at various time intervals and treated as described above. All experiments were performed at least in triplicate. Where data collection was commenced before the cleavage of one equivalent of substrate, no difference in rate was observed before and after the cleavage of one equivalent. The kinetic parameters, k_{cat} and K_m, were determined using Eadie–Hofstee plots (rate/[substrate] versus rate), and are presented in Table 1 as the mean ± SD.

Effect of magnesium ion concentration. The dependence of cleavage rates on magnesium ion concentration was investigated under ribozyme excess conditions in the range 2–200 mM $MgCl_2$. The appropriate amount of $MgCl_2$ was added to a solution of pre-annealed ribozyme–substrate to start the reaction. The depend-

ence on magnesium ion concentration was also investigated under conditions of substrate excess for several ribozymes. Separate solutions of ribozyme and substrate, at double the final concentrations of 100 nM and 3 μM, respectively, in the appropriate buffer and $MgCl_2$ concentration, were heated to 85°C for 3 min, centrifuged briefly, then cooled to 37°C prior to initiating the reaction by mixing equal volumes of the two solutions. The buffer used for these experiments was Mes, at either pH 5.37 or 6.45. The pH of the buffers was unaffected by the high $MgCl_2$ concentration as judged by an experiment in which the concentration of Mes buffer was varied; in the presence of 200 mM $MgCl_2$ there was no significant difference in the observed rate constants for cleavage of TAT S13 by TAT RA 6X2 at pH 5.37 at either 50, 100 or 200 mM Mes buffer. Another experiment was conducted in which the effect of $MgCl_2$ concentration on the cleavage rates of TAT S13 by TAT RA 6X2 was investigated at a constant ionic strength of 1.0 M, maintained by addition of NaCl. In calculating the amount of NaCl to be added, only the contribution of the ionised component of the buffer, and the $MgCl_2$ were considered. The ionic strength was calculated using the standard equation: $\mu = {}^1/_2\Sigma_i(c_iZ_i^2)$. In all these experiments the samples were treated as usual except that the formamide quenching solution contained EDTA sufficient to chelate all the Mg^{2+} ions present.

Effects of temperature and pH. Following the general procedure for ribozyme excess experiments, the effects of temperature and pH on the cleavage rates of the ribozymes were determined. The temperature dependence (5–45°C) was determined in 50 mM Mes buffer, pH 6.45 and 10 mM $MgCl_2$. The pH dependence was investigated at 37°C, 10 mM $MgCl_2$ in the following buffers at 50 mM; 5.37 and 6.45 (Mes) and 7.13, 7.57, 8.00, 8.39 and 8.77 (Tris).

Determination of k_{-1}. The measurement of the rate of dissociation of substrate from the ribozyme–substrate complex was determined by competition experiments. The ribozyme–substrate complex was pre-formed in the presence of buffer and 0.5 mM spermine but in the absence of $MgCl_2$, with ^{32}P-labelled substrate at a concentration many times the kinetically determined K_m for the reaction and ribozyme in excess. This mixture was pre-treated at 85°C as usual and the reaction at 37°C initiated either by addition of $MgCl_2$ to 10 mM, or $MgCl_2$ and a large excess (10–100-fold over labelled substrate) of unlabelled substrate. Samples were taken at various times and treated as described above. The reactions that had been initiated with addition of only $MgCl_2$ gave the usual exponential product–time curves, while the reactions initiated simultaneously with addition of $MgCl_2$ and excess unlabelled substrate displayed an initial burst of product followed by a linear increase of product with time. The ratio of the forward reaction rate to the rate of substrate dissociation, k_f/k_{-1}, is equal to $P_{\infty}S13/(P_{\infty}C - P_{\infty}S13)$, where $P_{\infty}S13$ is the amount of product produced by the rapid initial burst, (i.e. the amount of labelled substrate cleaved prior to dissociation from the ribozyme in the presence of excess unlabelled substrate), and $P_{\infty}C$ is the amount of product at $t = \infty$, in the reaction where no unlabelled substrate is added to the reaction mixture at the start of the reaction. k_f is the rate constant for the progression from ribozyme-substrate to ribozyme-product and may consist of a number of steps (*vide infra*).

RESULTS AND DISCUSSION

Prior to this study, we and others had shown that hammerhead ribozymes with DNA in the hybridising arms were more active than all-RNA ribozymes, with the effect of the DNA being to apparently increase the rate of cleavage (13–15) or to increase the rate of product dissociation (12). Since DNA–RNA complexes are generally less stable than RNA–RNA complexes (19), it seemed reasonable that DNA in the hybridising arms would influence the rate of product dissociation. However, it was not clear how DNA in the hybridising arms would affect the cleavage step, k_2, and so a more detailed investigation using two different systems was initiated.

Krüppel ribozymes

Under substrate-excess conditions, Kr RB cleaved the 13mer substrate Kr S13 at three times the rate of the all-RNA ribozyme Kr RA (Table 1, k_{cat}). The DNA-armed ribozyme (Kr RB) had a higher Michaelis constant (Table 1, K_m) than the all-RNA ribozyme, reflecting the expected lower affinity of the DNA-armed ribozyme for the substrate. Under ribozyme-excess conditions, both ribozymes displayed similar rate constants, intermediate between the two k_{cat} values (Table 1). The observed rate constant for Kr RB under these conditions was not increased at higher concentrations of Kr RB (9.5 μM) and Kr S13 (7.5 μM).

Table 1. Kinetic parameters for ribozymes

Ribozyme	Substrate excess		Ribozyme excess	
	k_{cat} (min^{-1})	K_m (nM)	k_{cat}/K_m ($min^{-1}\mu M^{-1}$)	k_{obs} (min^{-1})
Kr RA	4.4 (1.7)	440 (170)	10	8.9 (3)
Kr RA 6X2	–	–	–	>10
Kr RB	14.1 (5)	2290 (700)	6.2	7.6 (0.6)
Kr RB 6X2	–	–	–	>10
TAT RA	0.35 (0.02)	1080 (200)	0.32	0.24 (0.05)
TAT RA 6X2	6.3 (1.2)	320 (100)	20	>10
TAT RB	1.8 (0.5)	1300 (450)	1.4	4.5 (0.3)
TAT RB 6X2	5.2 (2)	900 (120)	5.8	9.9 (2)

Reaction conditions: pH 8.0, 10 mM $MgCl_2$, 37°C.
Values in parentheses are the standard deviations of at least two separate determinations.
k_{cat} and K_m are determined from turnover experiments where an excess of substrate is cleaved by a small amount of ribozyme.

TAT ribozymes

Under substrate-excess conditions, the DNA-armed ribozyme, TAT RB, was five times more efficient than the all-RNA ribozyme TAT RA (k_{cat} values of 1.8 and 0.35 min^{-1}, respectively; Table 1). The K_ms were not significantly different (1300 and 1080 nM) despite the expectation that the DNA-armed ribozyme would bind the substrate less avidly than the all-RNA ribozyme (19). Under ribozyme-excess conditions, TAT RB cleaved TAT S13 with a rate constant (k_{obs}) ~20-fold greater than TAT RA.

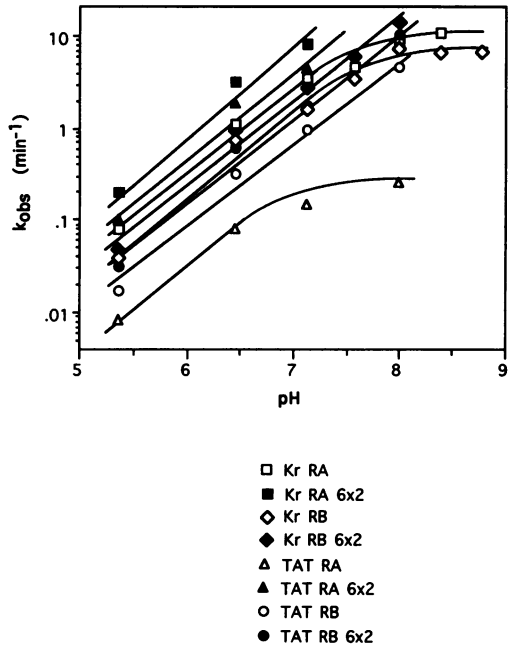


Figure 2. pH Dependence of k_{obs}. Reaction conditions: 37°C, 10 mM $MgCl_2$.

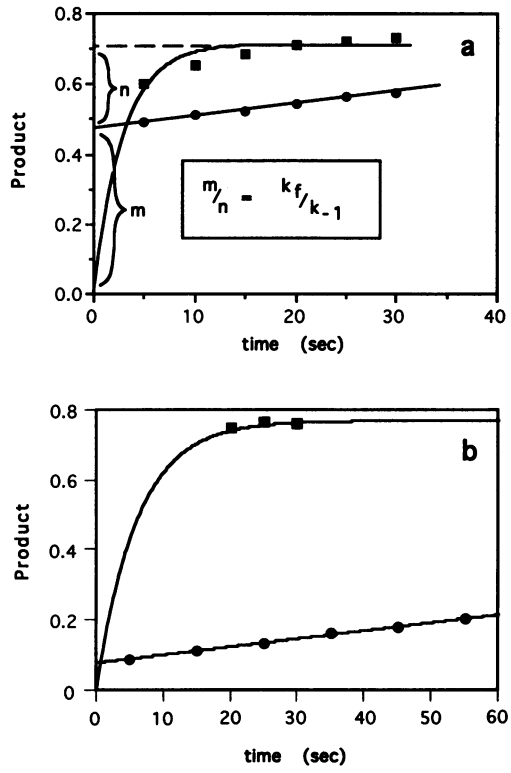


Figure 3. Experimental determination of k_{-1} for TAT S13. Reaction condidtions: pH 8.0, 37°C, 10 mM $MgCl_2$. (**a**) TAT RA 6X2, (b) TAT RB 6X2. ■ = control reactions without added unlabelled substrate; ● = reaction initiated simultaneously with $MgCl_2$ and excess unlabelled substrate. The curve fitted to the data in (**b**) is calculated assuming a rate constant of 9.9 min_{-1}.

Effect of shortening the hybridising arms

The effect that DNA in the hybridising arms has on the kinetic parameters clearly is not consistent for the two systems. To determine the reasons for the differences in behaviour, a more thorough study of the ribozymes was undertaken. In the Michaelis–Menten analysis of enzyme kinetics, $K_m = (k_{-1} + k_2)/k_1$, where the rate constants are as defined in Scheme 1. The relative values of K_m for TAT RA and RB seemed anomalous given that k_{-1} was expected to be considerably less for TAT RA than for TAT RB, and the expectation for k_2 was that if there was a difference it would be greater for TAT RB.

$$R + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RS \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RP_1P_2$$

Scheme 1.

In order to simplify the system, a ribozyme (TAT RA 6X2) which perfectly complemented the 13mer substrate was synthesised (this ribozyme is shown schematically in Fig. 1). The K_m of 320 nM for this ribozyme was substantially lower than the K_m of 1080 nM for the parent TAT RA (Table 1). The k_{cat} of 6.3 min^{-1} for TAT RA 6X2 was also much higher than k_{cat} for TAT RA (0.35 min^{-1}). Thus, in the turnover reaction of the long-armed TAT RA with TAT S13, binding of the substrate to the ribozyme appears to be increasing the apparent K_m and limiting the overall rate of the reaction.

k_{obs} for TAT RA 6X2 also increased substantially to >10 min^{-1} (Table 1), compared with 0.24 min^{-1} for TAT RA, and this now was greater than that observed for the DNA-armed ribozyme TAT RB (4.5 min^{-1}). Therefore, a shorter version of TAT RB, TAT RB 6X2, was synthesised and tested for cleavage ability. This shorter DNA-armed ribozyme displayed a small increase in k_{cat} (~3-fold) and in k_{obs} (~2-fold), and a small decrease in K_m, compared with the longer DNA-armed ribozyme TAT RB (Table 1). The two shortened TAT ribozymes, TAT RA 6X2 and TAT RB 6X2, now display, at pH 8, essentially identical k_{cat} values (6.3 and 5.2 min^{-1}), and K_ms which differ by a factor of 3 (320 and 900 nM, respectively). Their rate constants for the reactions performed with ribozyme in excess differ only 2-fold over the range of pH values (Fig. 2).

Short-armed versions of the Krüppel ribozymes were also prepared. These ribozymes, Kr RA 6X2 and Kr RB 6X2, displayed k_{obs} values only ~2-fold greater than their longer-armed analogues at the same pHs in ribozyme excess conditions (Fig. 2).

pH dependence

Since the cleavage reaction is dependent on the deprotonation of a functional group whose pKa is much greater than the pH of our experiments, (20 and *vide infra*), a first order dependence on [OH^-] would be expected if k_2 were rate limiting. In the pH range 5.37–8.77, the protonation states of the nucleotides in the ribozymes and substrates are not expected to vary significantly, and therefore the rates of hybridisation are not expected to be strongly dependent on pH in this range. Therefore the pH dependence of the reaction should be quite diagnostic. The dependence of cleavage rates on pH was therefore investigated

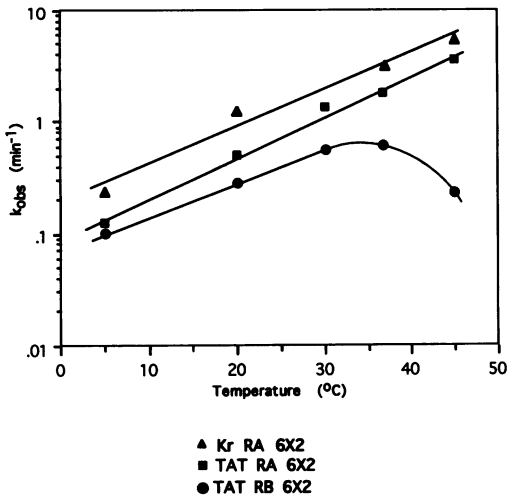


Figure 4. Temperature dependence for various ribozymes at pH 6.45 and 10 mM $MgCl_2$; ■ TAT RA 6X2; ● TAT RB; ▲ Kr RA 6X2.

for all the ribozymes. As shown by the data in Figure 2, all eight ribozymes displayed near first-order dependence on [OH^-] (gradient = 1.0) at low pHs, with gradients varying from 0.91 to 1.09. The ribozymes TAT RA 6X2, TAT RB, TAT RB 6X2, Kr RA 6X2 and Kr RB 6X2 retained this first-order dependence up to a pH where the rates approached the limit of measurement (~10 min^{-1}). TAT RA deviated from the first-order dependence on [OH^-] at ~pH 6.5. The long-armed Krüppel ribozymes RA and RB both deviated at ~pH 7.5, and showed similar rate constants at higher pHs. It is unlikely that the deviation from linearity at these pHs for these ribozymes reflects an approach to the pK_a of the metal-bound acidic (H_2O or 2′ OH) group, since the pK_a of that group is expected to be much higher (the pKa of a Mg^{2+}-bound H_2O or 2′ OH might be expected to be reduced by ~2 pK units relative to the uncoordinated ligand, i.e. to ~10–12) (20 and references therein). Also, we have observed a linear dependence of k_{obs} on pH, with close to unit slope up to pH 9.3 for a less-active hammerhead derivative (21), and other workers have observed the linear dependence for a ribozyme up to pH 8.9, albeit with a slope of only 0.7 (20).

A new reaction scheme

At low pHs, of the pairs of long and short ribozymes, only TAT RA and TAT RA 6X2 differ greatly in their k_{obs}; their rate constants differ by a factor of ~10. The origin of this difference is important in understanding the factors controlling the reactivity of these ribozymes. TAT RA has been synthesised a number of times with consistent results, which excludes the possibility that the difference is due to an aberrant synthesis. It seems unlikely that there is a real difference in k_2 of this magnitude between the two ribozymes, since once the substrate is bound the only difference between the complexes is the presence of four formally unpaired nucleotides on each end of TAT RA. Such a minor difference, well removed from the active site, would not be expected to have such a large effect on the cleavage rate constant. Also this scheme does not explain the deviation from the expected pH dependence above ~pH 6.5.

An alternative scheme in which a large proportion of the ribozyme is in a conformation (dimer or hairpin) which does not bind the substrate may account for the low k_{obs} and the deviation from expected pH dependency. Nuclease digestion experiments demonstrated that the hybridising arms of TAT RA are not readily accessible by single strand specific nucleases, which is consistent with the formation of a stable hairpin or dimer structure by this ribozyme. To test the possibility that the TAT RA–substrate complexes are not fully formed under the conditions of our experiments, rates of cleavage were measured over a wide range of conditions. There were no significant differences between k_{obs} values measured at low concentrations (3 μM TAT RA, 2 μM TAT S13) either in the presence or absence of 0.5 mM spermine, or at higher concentrations (11.8 μM TAT RA, 9.2 μM TAT S13). The observed rate constant at pH 6.45 (0.072 ± 0.007 min^{-1}) was independent of TAT RA concentration (3, 5, 10 and 20 μM) when the substrate concentration (2 μM) was kept constant. Additionally, when the ribozyme concentration was varied (10, 100 and 500 nM) with the substrate in excess (5 μM), the observed k_{cat} was constant at 0.06 ± 0.02 min^{-1} (pH 6.45) and 0.37 ± 0.03 min^{-1} (pH 8.00). The substrate concentration was, of course, varied over a wide range in the measurement of k_{cat} and K_m. Therefore under a wide variety of conditions it has not proven possible to increase the observed rate constants for cleavage of substrate by TAT RA. These observations demonstrate that the TAT RA–TAT S13 complex is fully formed under the conditions used to measure k_{obs} and the pH dependence. Thus although hairpin and/or dimer formation may be a factor in the higher than expected K_m for TAT RA, it does not explain the anomalously low values observed for the cleavage rate constants obtained with saturating substrate concentrations.

Since the complex is fully formed prior to initiation of the reaction and product dissociation is irrelevant under ribozyme excess conditions, the anomalously low activity for TAT RA must originate in a step after complex formation and before product dissociation. Scheme 1 is therefore too simplistic to explain these data. The step we have labelled k_2 in Scheme 1 must be a composite of at least two steps, a preliminary change followed by the cleavage step, k_2 (Scheme 2). The preliminary step, characterised by the rate constant k_c, occurs only after the addition of Mg^{2+} (because there is no burst of product observed), does not involve complete dissociation of the ribozyme–substrate complex (because some cleavage is observed without dissociation in the competition experiments) or any change in the stoichiometry of the complex (because it is independent of both ribozyme and substrate concentration). The step is normally kinetically invisible because there is no change in stoichiometry of the complex in going from RS′ to RS. At the moment it is not possible to say whether or not this step occurs for all ribozymes or is just a peculiarity of TAT RA. However, the same model could be used to explain the activities of Kr RA and Kr RB which plateau at pH 7.5 despite that fact that neither association nor dissociation can be rate limiting under the conditions of the experiment.

$$R + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RS' \underset{k_{-c}}{\overset{k_c}{\rightleftharpoons}} RS \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RP_1P_2$$

Scheme 2.

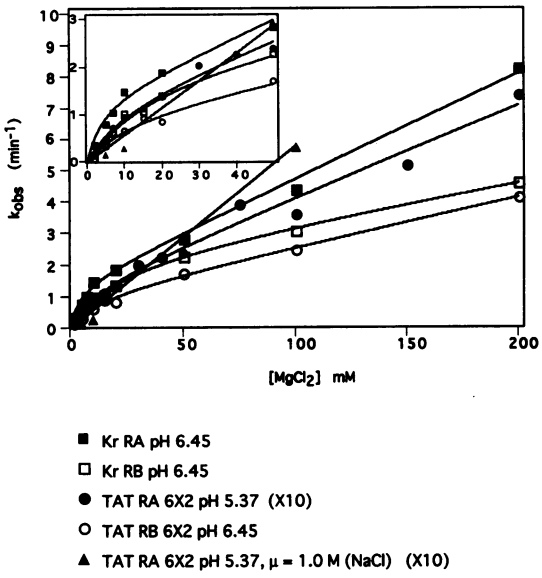
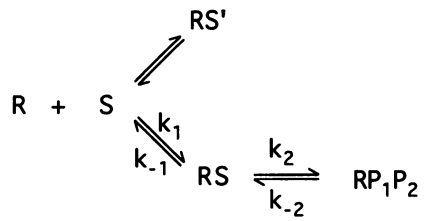


Figure 5. k_{obs} versus magnesium-ion concentration. Reaction conditions: 37°C. ■ Kr RA, pH 6.45; □ Kr RB, pH 6.45; ● TAT RA 6X2, pH 5.37; ○ TAT RB 6X2, pH 6.45; ▲ TAT RA 6X2, pH 5.37, μ = 1.0 M (NaCl). k_{obs} for TAT RA 6X2 are multiplied 10-fold for clarity. Inset graph shows more detail at low magnesium ion concentrations.

A scheme which involves a slow interconversion of an inactive ribozyme–substrate complex and its active form has been suggested earlier (22). In contrast to this work, in that publication the ribozyme and substrate were both >400 nt long and it is easy to envisage relatively stable inactive structures being rapidly formed and slowly equilibrating with the active form.

An alternate scheme (Scheme 3) which could possibly account for the observed low k_{obs} and the deviation from expected pH dependency can be dismissed since it would lead to a burst of product from the pre-formed RS, or if only the complex RS′ is formed in the absence of Mg^{2+}, then no cleavage would be expected without complete exchange of substrate, which is not the case (*vide infra*).

One possible manifestation of Scheme 2 could be that the inactive complex RS′ is required to partially dissociate in order to allow a conformational change to form the complex RS. The long hybridising arms in TAT RA may interfere in some way with the formation of the active complex, thereby limiting the rate of the reaction. At low pHs there is an equilibrium established between RS′ and RS, accounting for the 10-fold decrease in overall reactivity of the system while maintaining a first-order dependence on [OH^-]. At high pHs the rate of formation of RS



Scheme 3.

from RS′ becomes rate limiting, resulting in the observation of pH independence for the system.

Such a model for ribozyme cleavage (i.e. involving some element of partial substrate dissociation or flexibility) is also consistent with our observation that 21 nt substrates are cleaved much more slowly by the long armed ribozymes (2 × 10 nt hybridising arms) than are the 13 nt substrates even under single turnover conditions where the substrate and ribozyme are pre-annealed before the addition of magnesium to initiate the reaction (data not shown). If such a model for ribozyme cleavage is confirmed by further experimentation it will have very important implications for hammerhead ribozyme design.

Rates of substrate dissociation

The rates of dissociation of a number of ribozyme–substrate complexes were measured. This was performed in order to gain further insight into the reactivity of the ribozymes. The experiments also tested whether Mg^{2+} ions were able to bind rapidly to the pre-formed ribozyme–substrate complex. In these experiments, the pre-annealed complex of ribozyme with labelled substrate was allowed to react in the presence of a large excess of unlabelled substrate. If the labelled substrate dissociated prior to cleavage it would be diluted out by the unlabelled substrate. However, if the cleavage reaction was much faster than dissociation, then the labelled substrate would be cleaved at the same rate, and to the same extent, as observed in the absence of added unlabelled substrate. The ratio of labelled product initially formed in the presence of excess unlabelled substrate divided by the difference in initial product yield in the control (no excess substrate) and test (excess cold substrate) reactions is equal to the ratio of the forward and back reactions, (k_f/k_{-1}). Here k_f is the observed rate constant and is dependent on both the preliminary conformational change (k_c) and the cleavage step (k_2). This procedure is dependent on all the labelled substrate being complexed at the time of addition of $MgCl_2$ and unlabelled substrate, otherwise the value for the ratio (k_f/k_{-1}) will be under-estimated. Therefore, to favour complete complex formation, the following experimental conditions were used: in most cases the substrate concentration was >4-fold greater than the kinetically determined K_m for the reaction, the ribozyme concentration was typically 2-fold greater than the substrate concentration, spermine (0.5 mM) was added to the mix to assist annealing, and the Tris buffer was at 100 mM during the annealing phase. The experiments were performed in this manner to mimic the conditions of the ribozyme excess experiments, i.e. adding Mg^{2+} to preformed ribozyme–substrate to initiate the reaction.

The observation that, for all ribozymes, a measurable amount of cleavage of pre-annealed substrate occurs before dissociation, demonstrates that Mg^{2+} can bind to the pre-formed ribozyme–substrate complex and effect cleavage.

The results of these experiments are presented in Table 2 as k_f/k_{-1}, and examples of the experiments are shown in Figure 3. With the exception of the ribozyme, TAT RA 6X2, all values of k_f/k_{-1} are <1, indicating that upon addition of Mg^{2+} to pre-formed ribozyme–substrate complex, cleavage is slower than dissociation. For two ribozymes with high K_ms, the values of k_f/k_{-1} were checked at higher concentrations of ribozyme and substrate, to see if there were any changes. For TAT RA at 11.8 μM, and labelled substrate at 9.2 μM, the observed value of 0.021 was similar to that of 0.027 obtained at about one third of these concentrations (Table 2). Likewise, for TAT RB at 25 μM and

substrate at 10 μM, the observed value of 0.074 was similar to that of 0.065 observed at much lower concentrations. These competition experiments were performed at pH 8.0, and therefore k_{obs} at pH 8.0 was used to calculate k_{-1} from the k_f/k_{-1} data in Table 2 assuming that k_{obs} under these conditions is equal to k_f, ie. k_{-2} is negligible. The cleavage rate for TAT RA 6X2 was too fast to measure at pH 8.0 and therefore an estimate of k_{obs} was made by extrapolation from the data in Figure 2.

Table 2. Rates of dissociation and association between substrate and ribozymes (13mer substrates, pH 8.0, 10 mM $MgCl_2$, 37°C, 0.5 mM spermine)

Ribozyme	k_f/k_{-1}	k_{obs} (min^{-1})	k_{-1} (min^{-1})
Kr RA	0.4 (0.15)	8.9	22 (8)
Kr RB	0.035	7.6	217
TAT RA	0.027	0.24	9
TAT RA 6X2	2.0	37.6[a]	19
	9.2[b]	17.1[b,c]	1.9[b]
TAT RB	0.065 (0.015)	4.5	69 (16)
TAT RB 6X2	0.11	9.9	90

[a]Extrapolated to pH 8.
[b]At 20°C.
[c]Extrapolated from 20°C, pH 6.45 assuming pH/rate profile has a slope = 1.0

The rate constants for substrate dissociation from ribozyme pairs where identical base pairs are involved (TAT RA and TAT RA 6X2, and TAT RB and TAT RB 6X2) are very similar as expected. The substrate dissociation rate constants for DNA-armed ribozymes are 5–10-fold greater than for RNA-armed ribozymes.

The measured rates of substrate dissociation from the RNA-armed ribozymes studied here at 37°C (9–22 min^{-1}) are ~20–40-fold greater than observed by Fedor and Uhlenbeck (23) at 25°C, ~0.5 min^{-1}, for the same number of base pairs formed between ribozyme and substrate. However, the estimate we have made of k_{-1} at 20°C for TAT RA 6X2 (1.9 min^{-1}) is only ~4-fold greater, which could be due to differences in sequence. Hertel *et al.* (24) estimated a much slower k_{-1}, <0.003 min^{-1} at 25°C, pH 7.5, 10 mM $MgCl_2$, but in that case the number of base pairs formed between the ribozyme and substrate was 16 compared with the 12 here.

Dependence on temperature

The rates of reaction for the short-armed ribozymes Kr RA 6X2, TAT RA 6X2 and TAT RB 6X2 were measured over the temperature range 5–45°C, at pH 6.45. The results are shown in Figure 4. The rate constants for cleavage by the two all-RNA ribozymes, Kr RA 6X2 and TAT RA 6X2, increased with temperature in this range. An Arrhenius plot (ln k_{obs} versus 1/temperature) for each yielded activation energies for the reactions of 57 and 62 $kJmol^{-1}$, respectively. Earlier estimates of the activation energies for hammerhead cleavage reactions have ranged from 55 to 83 $kJmol^{-1}$ (24–27).

The rate constant for cleavage by the DNA-armed ribozyme TAT RB 6X2 increases to a maximum between 30 and 37°C, but declines rapidly thereafter due to limiting complex formation at this concentration and temperature (all the experiments were conducted at a substrate concentration of 2 μM).

Dependence on magnesium-ion concentration

The dependence on magnesium-ion concentration for activity was measured for the two long-armed Krüppel ribozymes, Kr RA and Kr RB, and the two short-armed TAT ribozymes, TAT RA 6X2 and TAT RB 6X2, with $MgCl_2$ concentrations ranging from 2 to 200 mM at pH 6.45 (pH 5.37 for TAT RA 6X2). The experiments producing the data shown in Figure 5 were conducted with ribozyme in excess of substrate, and the reactions were started by adding $MgCl_2$. In addition, for TAT RB 6X2, Kr RA and Kr RB, experiments were conducted with substrate in 30-fold excess over ribozyme (3 μM substrate, pH 6.45) and the reactions were initiated by mixing solutions of ribozyme and substrate which had been separately heat-denatured in buffer and $MgCl_2$. The k_{cat} values obtained under these conditions were close to the k_{obs} values found under conditions of ribozyme excess, and they displayed essentially identical magnesium-ion dependence (data not shown). The Mg^{2+} dependence for all the ribozymes is very similar. There is evidence of strong association between Mg^{2+} and the ribozyme–substrate complex with apparent K_ds of the order of 10 mM and unexpectedly there is a second phase in the Mg^{2+} dependence in which the rate constant for substrate cleavage increases linearly with Mg^{2+} concentration. The data in Figure 5 are fitted to the equation:

$$k_{obs} = \frac{(k_{max} * [Mg^{2+}])}{(K_d + [Mg^{2+}])} + (a * [Mg^{2+}])$$

The first term represents the expected tight binding, where k_{max} represents the limiting rate constant, in the presence of a single bound metal ion. The second phase is accounted for by the linear term since there is no evidence of saturation of this phase of the reaction. The apparent K_ds for Mg^{2+} binding, in mM, determined from the data in Figure 5, are as follows: TAT RA 6X2, 10 ± 9; TAT RB 6X2, 13 ± 5; Kr RA, 4 ± 3, and Kr RB, 21 ± 5. It is difficult to determine these apparent dissociation constants accurately because of the uncertainty associated with the slope and intercept of the following phase, but it is clear that the binding affinity of these ribozymes for Mg^{2+} is in the region observed previously (20,25,28,29).

The second phase of the Mg^{2+} dependence is unexpected, since in the majority of examples published to date the rates of cleavage appear to plateau out >30 mM (20,25,28,29). In those studies however, the authors did not investigate the Mg^{2+} dependence >50 mM and consequently may not have detected a second phase in the Mg^{2+} dependence had it been present. There is a single example in the literature of a ribozyme with a similar Mg^{2+} dependence to that described here: a DNA-armed ribozyme with a rate constant which continued to increase with increasing Mg^{2+} concentration up to 1.0 M (14). The analogous all-RNA ribozyme, however, did not behave in this fashion and its cleavage rate constants were independent of Mg^{2+} concentration >100 mM.

Like all the studies published to date, the effect of magnesium ion concentration on the cleavage activity of the ribozymes has been performed in the absence of ionic strength control. It is possible that the increase in rate constant observed at high $MgCl_2$ concentrations is due to an increase in ionic strength rather than an effect of magnesium ion concentration. The effect of $MgCl_2$ concentration on the cleavage activity of TAT RA 6X2 at a constant ionic strength of 1.0 M (maintained by addition of NaCl) was investigated. The high ionic strength (or high sodium ion concentration) has the effect of eliminating the curvature observed

at the low Mg^{2+} concentrations, Figure 5, but clearly the continuing increase in cleavage rate constants above 50 mM $MgCl_2$ is not simply due to an increase in ionic strength.

The biphasic Mg^{2+} dependence that we have observed is suggestive of the involvement of more than one metal ion in the chemistry of the cleavage process. This is consistent with theoretical considerations of the Mg^{2+}-catalysed reactions of phosphate esters, where it has been predicted that one metal ion binds to the 2′ OH to generate a metal bound alkoxide ion nucleophile and a second metal ion binds to the leaving group to stabilise the developing negative charge (30).

Other work

Taira and co-workers have shown in a number of publications (14,15,31,32) that a DNA-armed ribozyme with only five hybridising bases in each arm displays a k_{cat} 3–4-fold greater than that of its all-RNA analogue at pH 8.0. They also observed a similar increase in k_{cat} for an all-RNA ribozyme when the substrate was predominantly DNA (32). The cleavage activity of the all-RNA ribozyme is dependent on the apparent deprotonation of a functional group with a pK_a of 8.5 (30). If the DNA armed ribozyme did not display this dependence, then this may account for at least some of the difference.

The differences in K_m between all-RNA and DNA-armed ribozymes in this study are much smaller than the 65-fold difference observed by Shimayama *et al.* (31) where the K_m values for the DNA-armed and the all-RNA ribozymes were 1300 and 20 nM, respectively. The discrepancy probably relates to differences in sequence, since it has been reported that DNA/RNA hybrid duplexes are more RNA-like in structure and stability when the RNA strand of the hybrid is composed predominantly of purines, whereas the hybrid duplexes are more DNA-like if the DNA strand consists predominantly of purines (33). Our TAT substrate is 67% purine (eight out of 12 bases, excluding the formally unpaired, cleaved ribocytidine) and our Kr substrate is 50% purine (6/12), and thus the hybrid duplexes formed by these RNA substrates and their DNA-armed ribozymes are likely to be RNA-like in stability. In contrast, the substrate of Shimayama *et al.* (31) with sequence 5′ GCCGUCCCCCG 3′, is only 30% purine (3/10) and two of these purines are terminal; and so the DNA/RNA duplex formed by this substrate with their DNA-armed ribozyme would be expected to be more DNA-like, with a much lower stability relative to the RNA/RNA duplex formed with the all-RNA ribozyme, as was observed.

Conclusions/implications for ribozyme design

While this study contradicts previous suggestions that DNA-armed ribozymes will always display an enhanced k_{cat} compared with all-RNA ribozymes, they remain the preferred starting points for the development of exogenously-supplied therapeutic agents. First, DNA is significantly cheaper and easier to synthesise than RNA. Secondly, it is more resistant to nucleases, and can be further protected easily, for example, by phosphorothioate modification or 3′ terminal blocking. Thirdly, since DNA arms will hybridise less avidly to the target substrate, they may have increased specificity because the hybridising arms can be longer while still retaining competitive substrate dissociation rates. Finally, for any given length of hybridising arm, ribozymes with DNA, as opposed to RNA, arms are less likely to self-hybridise and form inactive structures.

In designing ribozymes for use *in vivo*, careful consideration must be given to the length and nature of the hybridising arms. In addition to considerations in the previous paragraph, the choice of arm length should include a consideration of how the arm length affects the rate of dissociation of substrate from the ribozyme relative to the rate of cleavage. *In vivo* where it may be substrate unfolding or accessibility that is rate limiting, it is important that the ribozyme, on binding the correct substrate, cleaves it before it dissociates and the substrate refolds or becomes inaccessible (for example by binding to protein). Thus the length of arms on the ribozyme (and the sequence, where there is a choice of target) should be chosen so that k_2 is at least comparable with k_{-1}. While selectivity arguments suggest that k_2 should be much lower than k_{-1} (34), a ribozyme chosen so that k_2 exceeds k_{-1} when complexed with its targeted substrate will have a lower k_2 and a higher k_{-1} when complexed with a mis-paired substrate, and therefore adequate selectivity may still be obtained. At this stage of the development of ribozyme therapeutics, where proof of efficacy is still the driving force, ribozymes should be given every chance to cleave their targets.

This work demonstrates the importance of understanding the fundamental processes which occur during ribozyme cleavage reactions. It has shown that, under different conditions, different rate-limiting steps control the overall reactivity and, consequently, changes to the ribozyme that appear to be minor can lead to order of magnitude changes in reactivity. The presence of DNA in the hybridising arms of hammerhead ribozymes does not, at least in these two well-studied examples, enhance the cleavage rate constant k_2. However, it does have an effect on substrate and product dissociation rates and can have a dramatic effect on the overall rate of reaction.

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