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REVIEW

Hammerhead ribozyme kinetics

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INTRODUCTION

The hammerhead ribozyme is a small RNA motif that self cleaves at a specific phosphodiester bond to produce 2′,3′ cyclic phosphate and 5′ hydroxyl termini (Hutchins et al., 1986; Forster & Symons, 1987a). The secondary structure of the hammerhead consists of three helices of arbitrary sequence and length (designated I, II, and III) that intersect at 15 nucleotides termed the catalytic core (Fig. 1A) (Forster & Symons, 1987b; Hertel et al., 1992). The X-ray crystal structures of two hammerhead ribozyme–inhibitor complexes revealed that the core residues fold into two separate domains and the helices are arranged in a Y-shape conformation with helix I and helix II forming the upper portion of the Y (Pley et al., 1994; Scott et al., 1995). Although the hammerhead is found as an intramolecular motif embedded in several RNAs in vivo (Symons, 1989), it can be assembled from two separate oligonucleotides (Fig. 1B) in three different arrangements (Uhlenbeck, 1987; Haseloff & Gerlach, 1988; Koizumi et al., 1988; Jeffries & Symons, 1989). In these bimolecular formats, the hammerhead effects RNA cleavage in a similar manner to a true "enzyme," proceeding through multiple rounds of substrate binding, cleavage, and product release (Uhlenbeck, 1987).

Because of its relatively small size, ease of synthesis, and its well-described structure and cleavage properties, the hammerhead has been useful for studying many facets of RNA structure and function. The major focus has been on understanding the mechanism by which the hammerhead catalyzes RNA cleavage. The role of specific functional groups in either binding or catalysis has been probed by incorporation of both natural and modified nucleotides into the core of the hammerhead (Bratty et al., 1993; Tuschl et al., 1995; McKay, 1996; Chartrand et al., 1997). In addition, the importance of metal ions in mediating cleavage has been studied extensively with the aim of establishing their roles in folding and catalysis (Dahm & Uhlenbeck, 1991; Perreault et al., 1991; Dahm et al., 1993; Grasby et al., 1993; Menger et al., 1996; Peracchi et al., 1997; Feig et al., 1998). The hammerhead ribozyme has also been used for the calibration of methods to probe more general properties of RNA such as folding and dynamics. These methods include a gel mobility shift assay (Bassi et al., 1995, 1996), transient electric birefringence (Amiri & Hagerman, 1994), fluorescence resonance energy transfer (FRET) (Tuschl et al., 1994), binding of bases to abasic sites (Peracchi et al., 1996), disulfide crosslinking (Sigurdsson et al., 1995), and UV crosslinking (Woisard et al., 1994; Wang & Ruffner, 1997). Finally, by forming an intermolecular complex with a substrate sequence embedded in a target mRNA, the hammerhead ribozyme in the I/III format is being pursued as a tool for gene inactivation inside cells (Haseloff & Gerlach, 1988; Christoffersen & Marr, 1995; Rossi, 1995). Because the only sequence in the target RNA required for cleavage is a U followed by C, A, or U, a hammerhead ribozyme can be designed to cleave virtually any RNA molecule.

Because essentially all experiments that involve the hammerhead require an analysis of the cleavage reaction, a basic understanding of the kinetic properties of the reaction is critical. The focus of this review is to describe the methods used to obtain a complete kinetic description of the bimolecular hammerhead ribozyme cleavage reaction and to summarize what has been learned from such an analysis. Emphasis is on the best-characterized I/III format hammerheads (Fig. 1), although many of the conclusions are valid for other hammerhead formats, as well as *cis*-cleaving hammerheads. Although the measurement and interpretation of the rate constants of the cleavage reaction follow the well-established principles of enzyme kinetics, there are several unusual properties of the hammerhead that

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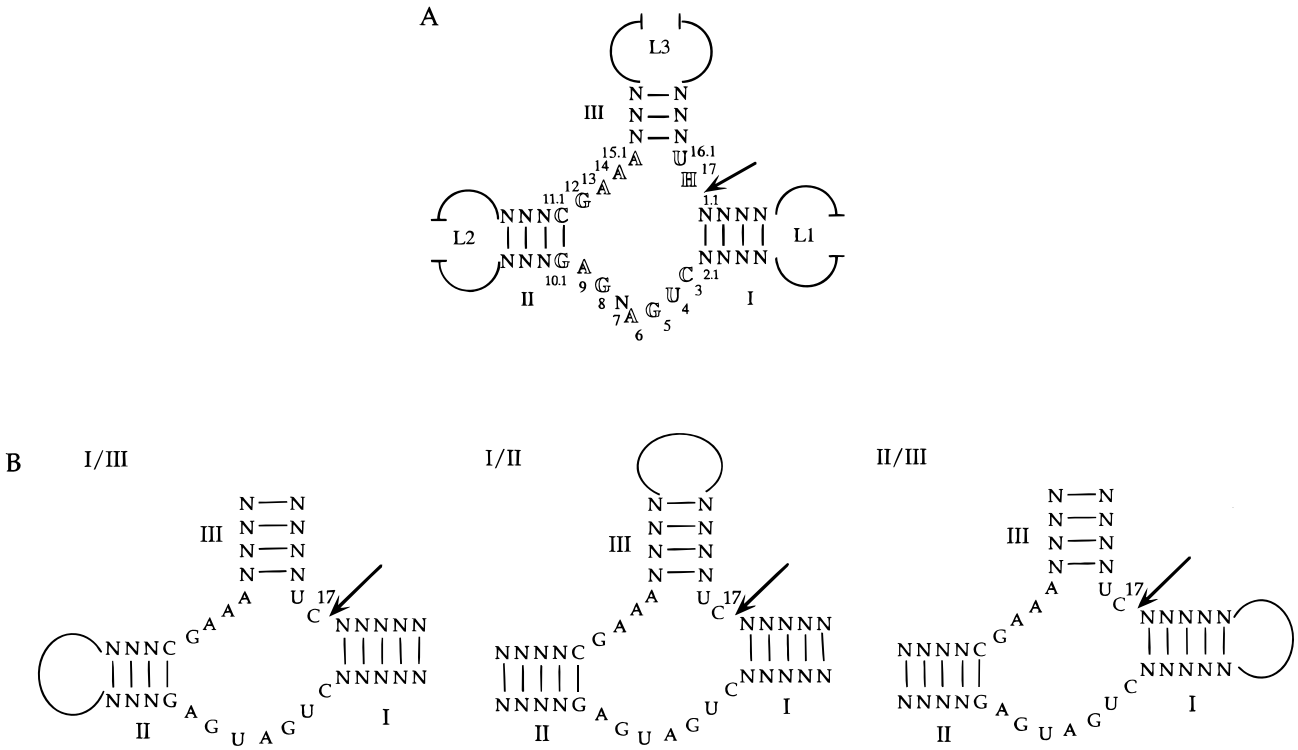


FIGURE 1. A: Consensus secondary structure of the hammerhead numbered according to (Hertel et al., 1992). The essential core nucleotides are designated in bold (H = A, U, C and N = nucleotide). The three loops (L1–L3) vary in length and sequence depending on where the hammerhead motif is embedded. Arrow represents the site of cleavage 3′ of position 17. **B:** Three bimolecular formats of the hammerhead designated by the helices through which the substrate binds the ribozyme.

make reiteration worthwhile. The general approach described herein can also be applied to other naturally occurring, small catalytic RNA motifs (Chowrira et al., 1993; Guo & Collins, 1995; Hegg & Fedor, 1995; Kawakami et al., 1996) as well as DNA oligonucleotides obtained by in vitro selection experiments that cleave RNA (Breaker & Joyce, 1994, 1995) and oligonucleotides containing tethered cleavage reagents (Bashkin et al., 1994). Elegant kinetic analysis of several of the larger catalytic RNAs have also appeared (Herschlag & Cech, 1990b, 1990a; Beebe & Fierke, 1994; Pyle & Green, 1994; Chin & Pyle, 1995), but they generally involve more complex kinetic schemes. A major conclusion of this review is that the kinetic and thermodynamic properties of all hammerheads can be understood in terms of the RNA helix-coil transition and the catalytic properties of the core residues. As a result, it is possible to predict the individual rate constants of the cleavage reaction for any hammerhead sequence under any buffer conditions with reasonable certainty. A second major conclusion of this review is that alternate conformations of the ribozyme, substrate, or ribozyme–substrate complex often profoundly affect one or more steps of the reaction pathway and thereby complicate interpretation of the observed cleavage rate. Many of the reported anomolies in hammerhead cleavage behavior are due to such alternate conformers that had not been identified. Other hammerhead topics such as the relationship of the three-dimensional structure to its function, its mechanism of catalysis, and its metal ion binding properties will not be discussed here. These and other topics have been reviewed elsewhere (Long & Uhlenbeck, 1993; Pan et al., 1993; McKay, 1996; Usman et al., 1996; Birikh et al., 1997).

THE HAMMERHEAD KINETIC PATHWAY—AN OVERVIEW

A minimal kinetic pathway has been established for the hammerhead cleavage reaction containing four main species, the ribozyme (E), substrate (S), ribozyme–substrate complex (E·S), and ribozyme–product complex (E·P1·P2) (Fig. 2) (Fedor & Uhlenbeck, 1992). In the I/III format, free ribozyme and substrate bind through helices I and III to form E·S. In the presence of magnesium or other divalent metal ions, the E·S complex can either cleave, producing E·P1·P2, or dissociate to free E and S. From E·P1·P2, the reaction either ligates back to E·S or proceeds with dissociation of each of the products from the ribozyme. Each of these steps is defined by an elemental rate constant (Fig. 2) (Fedor & Uhlenbeck, 1992; Hertel et al., 1994). It is possible that additional steps exist on the pathway that are too fast to be detected by the experimental methods currently

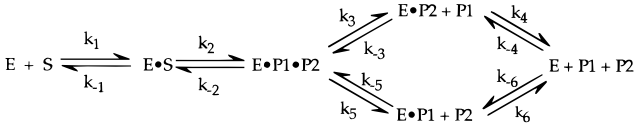


FIGURE 2. Minimal kinetic mechanism of the intermolecular hammerhead cleavage reaction. E is the ribozyme, S is the substrate, P1 is the 5′ product, and P2 is the 3′ product. Lowercase k's represent the elemental rate constants for each step of the minimal kinetic pathway.

used. Steps that have been proposed include: (1) conversion of E·S to a short-lived active complex with the attacking 2′ oxygen positioned in line with the scissile phosphodiester bond (Pley et al., 1994; Scott et al., 1995, 1996); (2) a large conformational rearrangement that involves docking of the two domains of the catalytic core (Peracchi et al., 1997); (3) a metal ion binding step (Long et al., 1995); or (4) a conformational switch from an inactive E·S to an active E·S (Bassi et al., 1995, 1996).

It is well known that many RNA sequences can adopt multiple alternate structures that are as stable as the native structure (Herschlag, 1995; Uhlenbeck, 1995). The addition of a single alternate equilibrium involving one of the species of the minimal hammerhead kinetic pathway can alter the kinetics of cleavage in several different ways. Both the rate of exchange and the overall equilibrium between the native and alternate structure can significantly alter the kinetic properties of the cleavage reaction. To give just one example, consider a situation in which an alternate conformation of E·S, termed [E·S]′, forms off of the main pathway (Fig. 3A). If the exchange rate is slow relative to the rate constant for cleavage (k_2) and the equilibrium constant results in, say, 40% of the complex being [E·S]′, the cleavage reaction will be biphasic with a fast rate, k_2, up to 60% product, followed by a slow rate reflecting the conversion of [E·S]′ to E·S. Very different behavior exists when the exchange rate is fast with respect to k_2. As before, the amount of active E·S available for conversion to E·P1·P2 is reduced by the fraction of [E·S]′ formed at equilibrium, however, a single, slower rate of cleavage will be observed that equals $(k_{conf'}/k_{conf})k_2$. Many other possible scenarios involving alternate structures can exist (Fig. 3B,C,D) and these species are not always easy to detect. The challenge is therefore to uncover these additional steps and to kinetically distinguish them from the steps of the minimal kinetic pathway. The easiest hammerheads to work with are obviously those that do not have alternate conformations of the reaction species. Several of these kinetically well-behaved or ideal hammerheads have been identified and steps can be taken to test whether sequences show such behavior (Fedor & Uhlenbeck, 1990, 1992; Heus et al., 1990; Hertel et al., 1994; Clouet-d'Orval & Uhlenbeck, 1996). Kinetically well-behaved

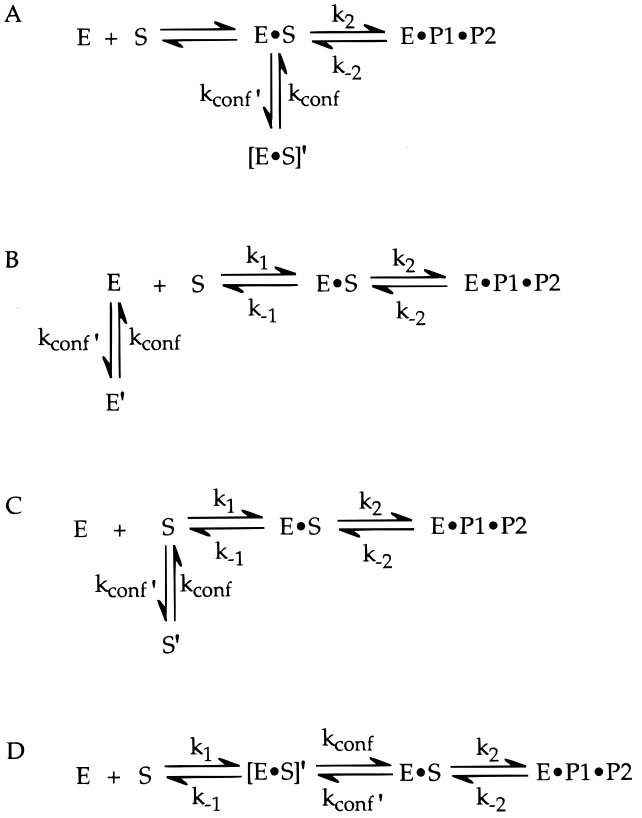


FIGURE 3. Possible alternate structures of species on the hammerhead reaction pathway. **A:** Schematic representation of an alternate conformation of E·S off of the minimal kinetic pathway. [E·S]′ is the alternate structure, k_2 is the rate constant for the chemical step, and k_{conf} is the rate constant for conversion of [E·S]′ to active E·S. **B:** An alternate conformation of E, E′, that must unfold to bind S. **C:** An alternate conformation of S, S′, that must unfold to bind E. **D:** An alternate conformation of E·S, [E·S]′, on the kinetic pathway that must convert to active E·S for cleavage to occur. k_{conf} and $k_{conf'}$ are the rates of conversion between the alternate structure and native structure.

hammerheads are the best to use for any type of biochemical or structural study; it is therefore critical to be able to detect hammerheads that exhibit nonideal behavior. In some cases, it may also be interesting to understand how an alternate conformation affects the reaction path.

MEASURING HAMMERHEAD CLEAVAGE RATES

Because the kinetics of hammerhead ribozyme cleavage are slow in comparison to many protein enzymes, the rates (<5 min^{-1}) have usually been measured by manual pipetting methods. Separation of the full-length radiolabeled substrate from the two cleaved products is readily accomplished by denaturing PAGE. The major advantage to this assay is that a large range of RNA concentrations can be used and that the products of the reaction can be quanti-

fied easily. Disadvantages of this assay include that it is discontinuous and thus relatively time consuming. FRET has been adapted for continuous measurement of some of the rate constants of the hammerhead cleavage reaction (Perkins et al., 1996). Here the focus will be on rates determined from the more widely applied gel separation method.

Two protocols are commonly used to perform the cleavage reaction. In the standard protocol, the ribozyme and substrate are heated separately at 95 °C in buffer without $MgCl_2$ for 1–2 min to disrupt any aggregates that may form during storage and then cooled to the reaction temperature. $MgCl_2$ is then added to both the ribozyme and substrate, incubated at the reaction temperature, and cleavage is initiated by adding the ribozyme–$MgCl_2$ mix to the substrate–$MgCl_2$ mix. Time points are taken and quenched in a stop solution containing denaturant and enough EDTA to chelate the magnesium. Occasionally, an annealing protocol is used where the ribozyme and substrate are heated together at 95 °C in the presence of buffer without magnesium, and subsequently cooled to the reaction temperature to promote annealing. $MgCl_2$ is added to initiate cleavage, and aliquots of the reaction are removed at specific times and quenched as before. The annealing protocol can be useful for isolating the chemical cleavage step under certain circumstances. However, a prevalent misconception is that annealing together a ribozyme and substrate that form stable helices necessarily bypasses the association step (Homann et al., 1994; Hendry & McCall, 1996; Hormes et al., 1997). This is only true at saturating amounts of ribozyme when binding is fast or if the reaction mixture is allowed to reach equilibrium prior to adding magnesium.

Substrate and product from each time point are subsequently separated by denaturing PAGE and quantitated using a phosphorimager. The fraction of product at time t, F_t, is calculated by dividing the amount of product by the amount of substrate plus product and the data is fit to the equation:

$$F_t = F_0 + F_\infty(1 - e^{kt}),$$

where F_0 is the fraction of product at zero time (which should be zero unless breakdown has occurred), F_∞ is the fraction of product at the endpoint of the reaction, and k is the rate of cleavage (Fersht, 1985). For proper analysis, the data set should include several time points in the linear phase of the reaction as well as additional time points to establish the endpoint of the reaction. A good fit to the equation thoughout the time course ensures that only a single rate constant is appropriate (Fig. 4A). Early hammerhead kinetic papers often analyzed cleavage data by plotting the natural log of the fraction of substrate as a function of time and obtaining a rate constant from the slope of the early, linear part of the curve (Ruffner et al., 1989; Fedor & Uhlenbeck, 1990, 1992; Fu & McLaughlin, 1992; Hendrix et al., 1995). Although this approach yields approximately the correct rate, it either ignores the endpoint altogether or attempts to correct the rate using the experimental endpoint. Because it introduces error into the measurement, this method is not recommended.

RIBOZYME EXCESS— THE SINGLE-TURNOVER REACTION

The hammerhead cleavage reaction has been studied as either a single-turnover reaction in which ribozyme is in excess over the substrate and a single cleavage event is followed, or as a multiple-turnover reaction in which multiple rounds of substrate binding, cleavage, and product release are monitored (Fig. 2). The advantage of studying the reaction under single-turnover conditions is that the substate binding and release steps can be studied separately from product binding and release. In addition, at saturating enzyme, the cleavage step can be separated from substrate binding. In this and following sections, the procedures used to measure the four elemental rate constants that define binding (k_1, k_{-1}) and the chemical step (k_2, k_{-2}) will be outlined and the methods used to detect the presence of alternate conformations of different reaction species will be described.

A convenient initial step for analyzing a new hammerhead sequence is to determine the cleavage rate using a trace concentration (low nM to pM) of labeled substrate and a saturating concentration of ribozyme. Saturation has been achieved if the cleavage rate does not change when the ribozyme concentration is increased by several increments over a factor of 10. For an ideal hammerhead under saturating conditions, every substrate molecule is expected to bind to a ribozyme and the observed rate of cleavage (k_{obs}) should equal the sum of the forward (k_2) and reverse rate constants (k_{-2}). For the hammerhead under most reaction conditions, k_2 is much (20–100-fold) faster than k_{-2}, so $k_{obs} = k_2$ (Hertel et al., 1994; Hertel & Uhlenbeck, 1995). However, for other ribozymes, such as the hairpin, k_{-2} can be quite fast (Hegg & Fedor, 1995) and a separate experiment is required to obtain k_2 (see below).

There are many potential outcomes for a simple cleavage time course at saturating ribozyme concentration. The examples shown in Figure 4B are simulated, but many of these outcomes exist in the literature. The first example represents a reaction time course for a well-behaved hammerhead under "standard" conditions of pH 7.5, 10 mM $MgCl_2$, and 25 °C. The extent of cleavage is 90% and the reaction rate is k_2 at 1.0 min^{-1}. The second example is a hammerhead that cleaves more slowly (0.18 min^{-1}), but the reaction eventually reaches completion (Hendry & McCall, 1995; Hertel et al., 1996,

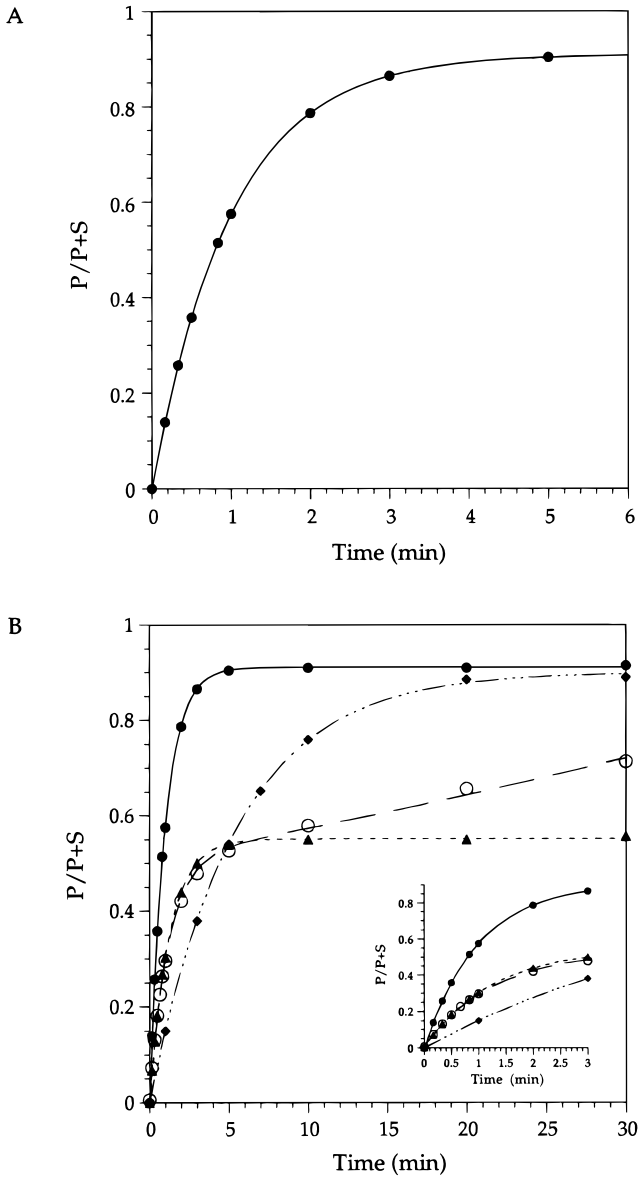


FIGURE 4. Example data for a single-turnover cleavage reaction at a saturating ribozyme concentration. **A:** Profile of the fraction product formed as a function of time fit to $(P/P + S) = (P/P + S)_0 + (P/P + S)_\infty(1 - e^{-kt})$, giving the rate $k = k_2 = 1.0\ min^{-1}$. **B:** Example outcomes for a single-turnover reaction at saturating ribozyme for a well-behaved hammerhead, $k = 1.0\ min^{-1}$ and $(P/P + S)_\infty = 0.9$ (●); a slow-cleaving hammerhead, $k = 0.18\ min^{-1}$ and $(P/P + S)_\infty = 0.9$ (♦); a hammerhead with a low extent of cleavage but normal rate of cleavage, $k = 0.8\ min^{-1}$ and $(P/P + S)_\infty = 0.5$ (▲); and a hammerhead having biphasic behavior, with a initial rate of $k = 0.8\ min^{-1}$ and a slow phase of $k = 0.2\ min^{-1}$ (○). The inset shows the first 3 min for each example.

1997). This behavior could be due either to a substrate in an inactive conformation that slowly converts to an active one, or to an alternate conformation of E·S that is either on or off the pathway as described earlier (Fig. 3). The third example in Figure 4B is a hammerhead that cleaves at a normal rate ($0.8\ min^{-1}$), but a low fraction of the substrate is cleaved (50%) (Fedor & Uhlenbeck, 1990). The explanations for this behavior include: (1) chemically impure substrate; (2) stable alternate conformations or aggregates of S; (3) a stable, inactive conformation of E·S off the cleavage pathway; (4) an inactive fraction of the ribozyme that binds the substrate stably; or (5) a rapid reverse reaction. The fourth example in Figure 4B is a reaction in which the kinetics are biphasic with an initial fast rate that corresponds to the normal cleavage rate (k_2) followed by a slow rate after 45% of the substrate is cleaved. This behavior may arise when an alternate conformation of E·S forms off the cleavage pathway and is in slow exchange with the active conformation, as described in the previous section. Other outcomes are possible that result from having a combination of more than one alternate equilibria.

Further experimentation is required to identify some of the potential causes for the reaction outcomes described above. The possibility of chemically impure substrate reducing the extent of cleavage can be tested by purifying the uncleaved substrate by denaturing PAGE and testing its ability to be cleaved in a second assay (Fedor & Uhlenbeck, 1990). If the cleavage profile is similar to the original, then the low extent of cleavage is not due to the presence of chemically impure substrate, but rather is due to an alternate substrate conformation that did not cleave in the original experiment but was destabilized during repurification. A lower extent of cleavage due to a decrease in the internal equilibrium constant ($K_{Int} = k_2/k_{-2}$) can be tested directly by measuring the extent of the reverse (ligation) reaction (see below).

One method to detect the presence of alternate stable conformations and aggregates of the ribozyme or substrate is to incubate separately the ribozyme and substrate oligonucleotides in the reaction buffer at various concentrations, and run the oligonucleotides into a nondenaturing gel containing cleavage buffer at the desired temperature. The temperature of the gel can subsequently be lowered to improve separation (Fedor & Uhlenbeck, 1990; Heus et al., 1990; Hertel et al., 1994; Clouet-d'Orval & Uhlenbeck, 1996). RNAs that form intramolecular secondary structures or intermolecular aggregates will often migrate as multiple bands on such gels. It is important to realize that a single species on a nondenaturing gel does not eliminate the possibility that alternate conformations are present (Heus et al., 1990). Poor separation or fast exchange between the native and alternate structure can lead to a single species on the gel despite kinetic discrepancies. Other methods that can be used to identify alternate structures include structure mapping under the conditions of the cleavage assay (Hodgson et al., 1994), thermal denaturation experiments (Ruffner et al., 1989; Yang et al., 1990), and NMR spectroscopy (Heus et al., 1990). Adding RNA chaperones such as HIV nucleocapsid protein to a cleavage reaction can be used to detect the presence of alternate conformations be-

cause they can lower the free energy barrier between the alternate and native structures (Tsuchihashi et al., 1993; Herschlag et al., 1994; Herschlag, 1995).

An alternate conformation of E·S, [E·S]′, which may be either on (Hendry & McCall, 1995; Hertel et al., 1996) or off the pathway and exchanges with E·S at a rate similar to the cleavage rate is not detected as easily. Because the rate of the chemical step is pH dependent and, in most cases, conformational changes are not influenced by pH, experiments at different pHs may aid in distinguishing these two possibilities. At a high pH where the rate of the chemical step is fast, the rate of conversion of [E·S]′ to E·S may become the rate-limiting step of the reaction. If [E·S]′ is on the pathway, then the observed rate of cleavage will be dictated by the rate of conversion of [E·S]′ to E·S and the reaction will eventually reach completion. If [E·S]′ is off the pathway, the reaction profile will become biphasic, having a burst of product formation from the active fraction of E·S followed by a slower phase for the conversion of [E·S]′ to E·S.

For a kinetically well-behaved hammerhead, the rate measured at saturation represents the rate constant for the chemical step (k_2). The value of k_2 is independent of the way in which the hammerhead is assembled. Similar k_2 values have been reported for all three hammerhead bimolecular formats (Fedor & Uhlenbeck, 1990; Heidenreich & Eckstein, 1992; Chartrand et al., 1995; Clouet-d'Orval & Uhlenbeck, 1996) as well as a *cis*-cleaving hammerhead (Long & Uhlenbeck, 1994). In addition, k_2 varies with buffer conditions in a similar manner for all hammerheads. It has been shown for several different hammerhead sequences that the log of the cleavage rate constant increases in linear proportion with pH (Dahm et al., 1993; Hertel & Uhlenbeck, 1995; Burgin et al., 1996; Clouet-d'Orval & Uhlenbeck, 1996). The cleavage rate also increases with increasing metal ion concentration in a similar fashion for several hammerheads, saturating between 0.1 and 0.5 M $MgCl_2$ (Dahm & Uhlenbeck, 1991; Koizumi & Ohtsuka, 1991; Perreault et al., 1991; Yang et al., 1992; Dahm et al., 1993; Grasby et al., 1993). Finally, the temperature dependence of k_2 gives a linear Arrhenius plot with a similar slope between 4 °C and about 40 °C (Uhlenbeck, 1987; Hendry et al., 1995; Hertel & Uhlenbeck, 1995; Takagi & Taira, 1995; Clouet-d'Orval & Uhlenbeck, 1996) for at least five different hammerheads.

Table 1 compares k_2 values for different hammerhead sequences from the literature and adjusts them to

TABLE 1. k_2 or k_{cat} values for several hammerheads from the literature.

Hammerhead	k_2 or k_{cat} (min^{-1})	R or S excess	Reaction conditions pH/mM $MgCl_2$/°C	Adjusted k_2 (min^{-1})[a]	Reference
HH6	1.5	S	7.5/10/25	1.5	(Fedor & Uhlenbeck, 1990)
HH6	2.3	S	7.5/10/25	2.3	(Williams et al., 1992)
HH6	1.5	R	7.5/10/25	1.5	(Fu et al., 1994)
HH9	1.5	S	7.5/10/25	1.5	(Fedor & Uhlenbeck, 1990)
HH8	1.4	R	7.5/10/25	1.4	(Fedor & Uhlenbeck, 1992)
HH13	1.2	R	7.5/10/25	1.2	(Fedor & Uhlenbeck, 1992)
HH14	1.9	R	7.5/10/25	1.9	(Fedor & Uhlenbeck, 1992)
HH15	2.0	R	7.5/10/25	2.0	(Fedor & Uhlenbeck, 1992)
RE161	2.3	S	7.5/10/37	0.9	(Heidenreich & Eckstein, 1992)
RE5	1.0	S	7.5/10/37	0.4	(Heidenreich & Eckstein, 1992)
RE115	1.4	S	7.5/10/37	0.6	(Heidenreich & Eckstein, 1992)
Kr S13	8.9 ± 3	R	8/10/37	1.2	(Hendry & McCall, 1995)
GH RA6/6	1.6	R	8/10/30	0.4	(Hendry & McCall, 1996)
HH16	0.95	R	7.5/10/25	0.95	(Hertel et al., 1994)
HTF	2.4	R	7.5/10/37	1.0	(Jankowsky & Schwenzer, 1996)
RNA HH	2.2	R	7.5/10/25	2.2	(Pley et al., 1994)
All RNA	2.6	S	7.5/10/37	1.0	(Perreault et al., 1991)
R32/R11	0.086	R	6/25/25	1.1	(Sawata et al., 1995)
R32/11	4.0	S	8/25/37	0.6	(Shimayama et al., 1993)
GUA	2.4	S	8/25/37	0.4	(Shimayama et al., 1995)
R1	0.72	R	7.5/10/25	0.72	(Sigurdsson et al., 1995)
S13	2.8	S	7.4/20/30	1.6	(Slim & Gait, 1991)
antiHIV	25	S	8/45.5/37	1.1	(Woisard et al., 1994)
R_{RN}/S_{FI}	2.4	S	7.3/20/37	1.0	(Perkins et al., 1996)
RPI.1435	1.5	R	7.5/10/37	0.6	(Burgin et al., 1996)
RZ1 (II/III format)	3.4	S	8.5/50/30	0.3	(Chartrand et al., 1995)
HHα5 (I/II format)	2.5	R	7.5/10/25	2.5	(Clouet-d'Orval & Uhlenbeck, 1996)
HHα7 (I/II format)	0.8	R	7/10/25	2.0	(Stage-Zimmermann & Uhlenbeck, 1998)

[a] k_2 and k_{cat} were adjusted to a standard set of conditions of pH 7.5, 10 mM $MgCl_2$, and 25 °C using the pH and magnesium dependence of k_2 for HH8 (Dahm et al., 1993), and the pH and temperature dependence of k_2 for HH16 (Hertel & Uhlenbeck, 1995).

standard conditions of pH 7.5, 10 mM $MgCl_2$, and 25 °C when necessary. The pH rate profile, $MgCl_2$ dependence, and temperature dependence data for two well-characterized hammerheads (HH8 and HH16) were used as the guidelines for these adjustments (Dahm et al., 1993; Hertel & Uhlenbeck, 1995). For example, a hammerhead cleavage rate of 1.6 ± 0.6 min^{-1} measured at pH 8 and 30 °C (Hendry & McCall, 1996) was reduced by a factor of 3 for pH and a factor of 1.5 for the temperature, resulting in a 4.5-fold slower adjusted k_2 of ~0.4 min^{-1}. Although the pH, magnesium, and temperature effects appear to be independent and are similar for several different hammerheads, such adjusted values should be considered only as approximations. Nevertheless, for 24 different hammerheads with rate constants measured in seven different laboratories, the adjusted k_2 values fall within the range of 0.4–2.5 min^{-1} (Table 1). This striking consistency demonstrates that the rate of the chemical step for the hammerhead ribozyme is independent of the sequence and length of its helices, and that therefore the conserved core must be responsible for cleavage.

THE LIGATION REACTION

The rate constant for ligation, k_{-2}, can only be measured for hammerheads that form a stable ribozyme–product complex (Hertel et al., 1994). The reaction protocol is very similar to that for the forward cleavage rate with the starting complex being E·P1·P2 rather than E·S. Because the rate of approach to equilibrium from either the forward or reverse direction is dominated by k_2 for the hammerhead (Hertel et al., 1994), k_{-2} must be calculated from the value of the internal equilibrium $K_{int} = k_2/k_{-2}$, which is equal to the ratio of substrate to products at equilibrium. This is best determined approaching the equilibrium from the product side of the reaction. k_{-2} has only been determined for a few hammerhead sequences but, in all cases, is about 100-fold slower than the forward cleavage rate constant (Hertel et al., 1994, 1997; Stage-Zimmermann & Uhlenbeck, 1998). As is the case for the cleavage rate constant, k_{-2} is both temperature and pH dependent (Hertel & Uhlenbeck, 1995).

TWO KINETIC REGIMES

The hammerhead ribozyme can fall into one of two kinetic regimes that are defined by the relationship between the dissociation rate, k_{-1}, and the rate constant for cleavage, k_2 (Herschlag, 1991). The first regime is when k_{-1} is much faster than k_2 such that the E·S complex is in rapid equilibrium with free E and S. The second regime is when k_{-1} is very slow compared to k_2 so that no pre-equilibrium occurs and every substrate that binds the ribozyme goes on to cleave. Because, as discussed above, k_2 is a constant for all hammerheads (in a given buffer), the major factor that determines the kinetic regime of a given hammerhead is the value of k_{-1}, which is related to the stability of helices I and III.

To determine which regime a given hammerhead falls into and to obtain information on k_{-1}, a pulse-chase experiment is performed. The protocol for this experiment, described in more detail elsewhere (Fedor & Uhlenbeck, 1992; Werner & Uhlenbeck, 1995; Clouet-d'Orval & Uhlenbeck, 1996), involves combining an excess, saturating concentration of ribozyme with trace-labeled substrate and allowing the reaction to proceed for a certain time, t_1, long enough such that all of the substrate is bound to the ribozyme (typically less than 1 min). After time t_1, the reaction is chased either by addition of a large excess of nonradioactive substrate or by dilution with reaction buffer, and the time course of the cleavage reaction is monitored. As a result of the chase, any labeled substrate that falls off the ribozyme during the chase period is unable to rebind and therefore is not cleaved. Several times of incubation prior to the chase (t_1) should be tried to verify that the result obtained is independent of this parameter. The dilution method is preferred over the nonradioactive substrate chase because the latter can lead to an abnormally fast substrate dissociation rate (Werner & Uhlenbeck, 1995). In parallel, two control reactions are performed, one omitting the chase (Fig. 5, open circles), and a second in which the chase is added at $t_1 = 0$ and thereby tests

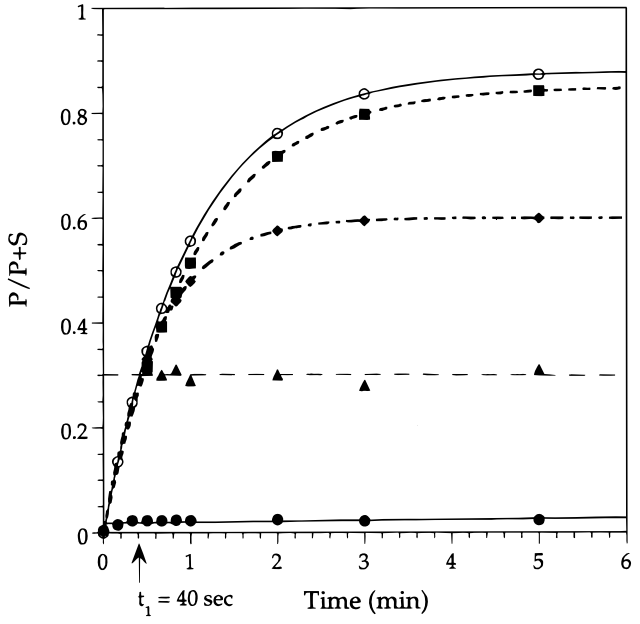


FIGURE 5. A pulse-chase experiment. Arrow labeled t_1 indicates the time at which the reaction was diluted. Two control reactions were run in parallel: the rate of the chemical step measured without a dilution, k_2 = 1.0 min^{-1} (○); and a reaction where the dilution was added prior to starting cleavage (●). The three possible outcomes are the first kinetic regime where $k_{-1} \gg k_2$ (▲); the second kinetic regime where $k_{-1} \ll k_2$ (■), and a case where $k_{-1} = k_2 = 0.8$ min^{-1}, so k_{obs} = 1.6 min^{-1} (♦).

whether the chase was effective at preventing rebinding of the labeled substrate (Fig. 5, solid circles).

This pulse-chase experiment effectively defines the two kinetic regimes for the hammerhead ribozyme. If no additional cleavage is seen after the chase is added, $k_{-1} \gg k_2$ and the first regime holds true (Fig. 5, triangles). If cleavage is not affected by the chase, $k_{-1} \ll k_2$ and the second regime fits (Fig. 5, squares). Certain hammerheads have a rate of substrate dissociation on the order of the rate of chemistry, $k_{-1} \cong k_2$ so that cleavage is seen during the chase period (Fig. 5, diamonds). In this relatively unusual case, k_{-1} can be determined directly from the chase experiment by one of two methods (Fedor & Uhlenbeck, 1992). For some hammerhead sequences, the kinetic regime can be changed by altering the pH of the reaction and thereby changing k_2 (Werner & Uhlenbeck, 1995; Clouet-d'Orval & Uhlenbeck, 1996). Because k_{-1} is not expected to depend on pH, changing the pH effectively alters the relative values of the two rate constants and potentially creates regime-one conditions.

Determining the kinetic regime for a hammerhead by the pulse-chase experiment defines the strategy to obtain k_1 and k_{-1}, the elemental rate constants for the binding step. For hammerheads in the first regime, k_1 and k_{-1} cannot be determined directly, although $K_d = k_{-1}/k_1$ is easily obtained (see next section). For hammerheads in the second regime, k_1 can be obtained by measuring the rate of cleavage at subsaturating ribozyme concentration where binding is rate-limiting. Under these conditions, $k_{obs} = k_1[R]$. The value of k_1 has only been measured for a limited number of hammerheads, but a value between 10^7 and 10^8 M^{-1} min^{-1} is usually obtained (Fedor & Uhlenbeck, 1992; Hertel et al., 1994). This value is similar to k_1 values reported for simple RNA duplexes in high concentrations of monovalent cation (Pörschke & Eigen, 1971; Pörschke et al., 1973; Nelson & Tinoco, 1982). However, k_1 values of DNA helices in buffers containing $MgCl_2$ are often faster (Williams et al., 1989), suggesting that faster hammerhead association rates may be possible.

DETERMINING E·S STABILITY

For those hammerheads in the first kinetic regime, the K_d for E·S can be obtained from a ribozyme saturation experiment where the rate of cleavage is measured over a range of ribozyme concentrations from low-nanomolar to mid-micromolar (Fedor & Uhlenbeck, 1992). The substrate concentration should be kept at least 10-fold lower than that of the lowest ribozyme concentration so that the free ribozyme concentration approximates the total ribozyme concentration. Either the annealing or nonannealing protocol can be used for this experiment because there is a rapid pre-equilibrium between E, S, and E·S. The observed rate of cleavage, k_{obs}, is plotted as a function of ribozyme concentration and fit to a binding equation to determine the rate at saturating ribozyme (k_{max}) and K_d. For hammerheads in the first kinetic regime, the value of K_d can also be determined in a *substrate* saturation experiment where the rate of cleavage is measured over a range of substrate concentrations and a low ribozyme concentration (Fedor & Uhlenbeck, 1990). In this case, many moles of substrate are cleaved by each ribozyme in a "multiple-turnover" reaction. Because $k_{-1} \gg k_2$, the E·S complex is in rapid equilibrium with free E and S and therefore the reaction behaves as a Michaelis–Menten enzyme and the saturation experiment can be analyzed by an Eadie–Hofstee plot. In this case, $K_M = (k_{-1} + k_2)/k_1$ reduces to $K_d = k_{-1}/k_1$. Because, for a regime-one hammerhead, the rates of product release are always faster than k_{-1}, $k_{cat} = k_2$ in such a substrate saturation experiment. In at least one case where it was carefully tested, the values of k_{max} and K_d obtained from a ribozyme saturation experiment agreed well with k_{cat} and K_M determined by a substrate saturation experiment (Fedor & Uhlenbeck, 1992).

It is important to point out that, for hammerheads in the second kinetic regime, either a ribozyme or a substrate saturation experiment does not give K_d or, indeed, any information about E·S stability, although the data will often superficially resemble a binding curve. This is because the cleavage rate at subsaturating ribozyme does not reflect the fraction of ribozyme bound to substrate at equilibrium, but rather reflects the fraction of ribozyme that binds substrate during the incubation time chosen and therefore is governed by k_1. E·S stability for hammerheads in the second regime can, however, be estimated by using the overall reaction equilibrium (Hertel et al., 1994).

Three example outcomes for a ribozyme saturation experiment are illustrated in Figure 6. The first example is for a well-behaved hammerhead that does not have any alternate conformations on its reaction pathway. The k_{max} is 0.95 min^{-1}, representing the rate of chemistry, and the K_d is 50 nM. The second example represents a ribozyme with a K_d of 60 nM, similar to that of the first hammerhead, but its k_{cat} is slow at 0.3 min^{-1}. This type of behavior may either reflect an alternate conformation of E·S on the reaction pathway (Fig. 3D), or an alternate conformation of S that binds E, forming an inactive E·S′ complex. Example three represents a hammerhead with a K_d of 620 nM and a k_{max} of 0.85 min^{-1}. This hammerhead may have very stable alternate conformations or aggregates of the ribozyme, E′, that reduce the amount of active E available to form active E·S (Fig. 3B). Thus, increasing the ribozyme concentration eventually provides enough active ribozyme to reach the maximal rate. Alternatively, the increase in K_d may be due to slow assembly of the ribozyme–substrate complex because conversion of inactive S′ to active S is required for binding (Fig. 3C).

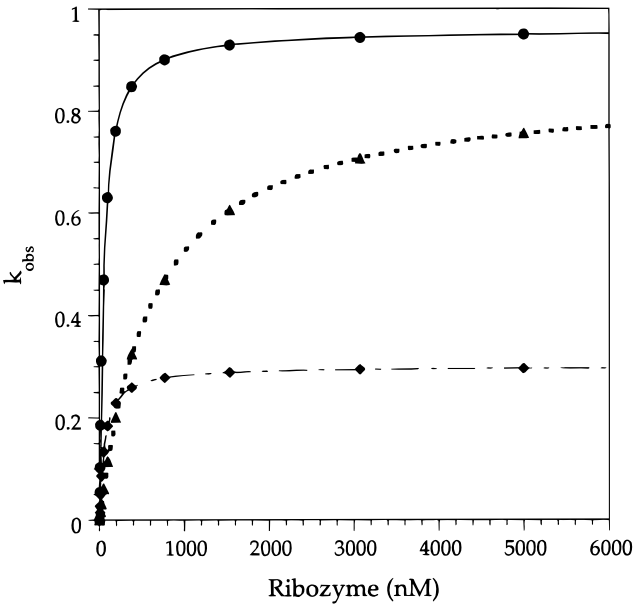


FIGURE 6. Ribozyme excess saturation experiment for a regime 1 hammerhead. Plot of k_{obs} as a function of ribozyme concentration fit to $k_{obs} = (k_{max} \times [R])/([R] + K_d)$ to obtain k_{max} and K_d with three example outcomes. An ideal hammerhead with a $k_{max} = 0.95$ min^{-1} and $K_d = 50$ nM (●), a hammerhead with a reduced $k_{max} = 0.3$ min^{-1} and $K_d = 60$ nM (♦), and a hammerhead having a high K_d, $k_{max} = 0.85$ min^{-1} and $K_d = 620$ nM (▲).

Hertel and coworkers recently compared the experimentally determined free energies for E·S stability for nine well-behaved hammerheads with the free energy of the corresponding uninterrupted helix I–III as calculated using the well-established rules for RNA duplex stability (Turner et al., 1988; Serra & Turner, 1995). A good correlation between the predicted and mesured free energies was observed when a free energy of +3.1 kcal/mol was assigned to the essential core nucleotides and stem loop II, which interrupt the perfect I–III helix (Hertel et al., 1994; K.J. Hertel, T.K. Stage-Zimmermann, G. Ammons, & O.C. Uhlenbeck, in prep.). This analysis permits the ΔG° and thus the K_d for the E·S complex of any hammerhead sequence to be estimated by subtracting the experimentally determined core energy from the calculated helix energy.

THE PRODUCT BINDING EQUILIBRIA

When the hammerhead helices are short, product oligonucleotides rapidly dissociate from the ribozyme after cleavage and thus do not influence the cleavage rate. However, when the helices are sufficiently long for the products to remain stably bound, the overall cleavage rate is affected. When ribozyme is in excess, stably bound products only slightly increase the rate of observed cleavage and slightly decrease the extent of reaction due to the slow reverse reaction. However, when substrate is in excess, stable products can dramatically influence the cleavage rate because their release becomes rate-limiting. Because product RNA duplexes as short as five base pairs can have dissociation rates at 25 °C slower than 1 min^{-1}, many hammerheads will show slower cleavage rates when measured in substrate excess than when measured in ribozyme excess. Indeed, there are numerous examples in the literature where the multiple-turnover cleavage rate at saturation was not assigned to a specific step of the reaction and the rate determined probably reflected product release (Koizumi et al., 1989; Goodchild, 1992; Hendry et al., 1992; Paolella et al., 1992; Slim & Gait, 1992; Taylor et al., 1992; Hendrix et al., 1995; Holm et al., 1995).

A burst experiment is useful for determining whether the rate-limiting step for a hammerhead reaction is k_2 or one of the product release steps. A burst reflects the fast appearance of products during the first turnover of the ribozyme that is followed by a slower appearance of products representing the subsequent rate-determining step of the pathway. Experimentally, a burst is easiest to detect when the substrate concentration is sufficiently high to be saturating throughout the experiment and a 3–10-fold excess of substrate over the ribozyme is used. Figure 7 gives two simulated data sets for a hammerhead that is limited by product release when a ratio of substrate to ribozyme of 10:1 was used. If all of the ribozyme can participate in the reaction, the formation of 10% product is equivalent to one turnover. In Figure 7A, several time points were taken in the first turnover, clearly revealing the burst of product formation. The rates of each phase as determined from this data set are 1.1 min^{-1} for the burst reflecting k_2 followed by 0.67 min^{-1} for subsequent turnovers reflecting release of one of the products. This burst behavior is more difficult to detect if insufficient data points are taken during the first turnover (Fig. 7B). Its existence can only be surmised by showing that the steady-state rate does not extrapolate to the origin at zero time. Similarly, if the substrate is in greater excess, not all the ribozyme is active, or if saturation is not maintained throughout the reaction, the burst phase may also be difficult to detect. This example stresses the importance of carefully defining the first turnover especially when the rate of product release may be close to the rate of the chemical step.

The absence of a burst in a multiple-turnover reaction is indicative of k_2 being rate-limiting, although this should be verified by comparison with k_2 measured under single-turnover conditions. If a burst is detected, the rates of product dissociation for both products can be measured directly by a pulse-chase method and analysis by nondenaturing gel electrophoresis as described in Hertel et al. (1994). The dissociation of the two products from the ribozyme follows the order of their affinity to the ribozyme with the less stable prod-

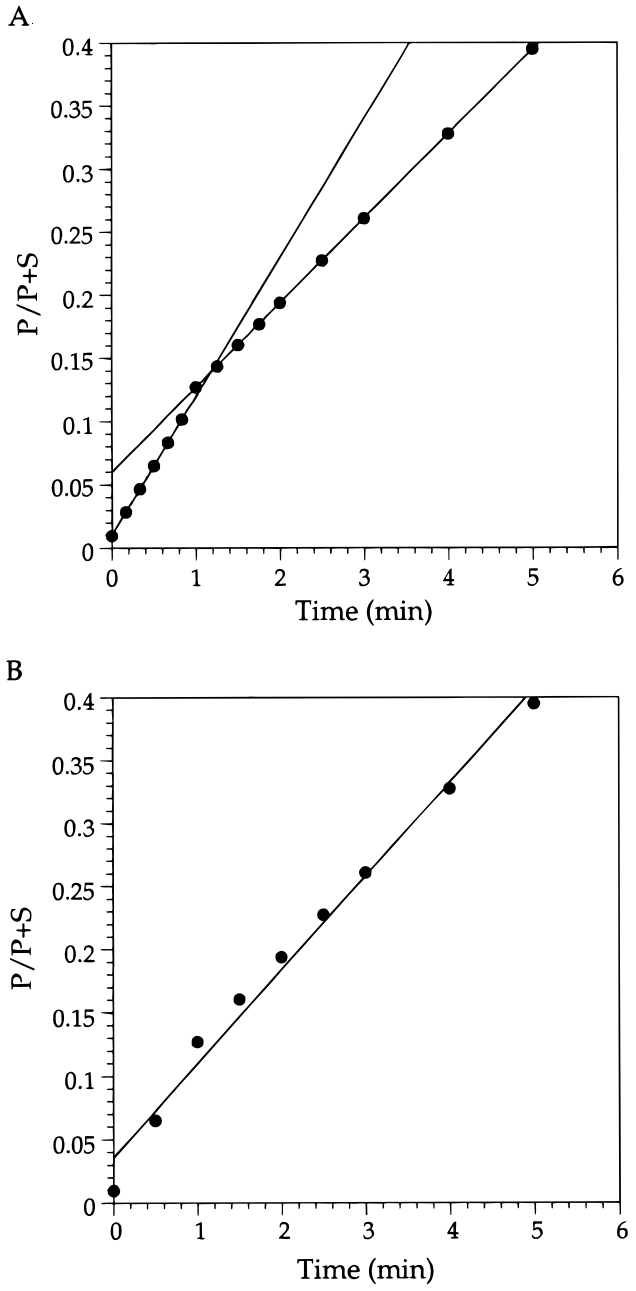


FIGURE 7. Burst experiment. **A:** Substrate excess experiment at saturating substrate for a hammerhead that is limited by product release. The data are for a 10:1 ratio of S:E and the first turnover is clearly defined by several time points. The rate of the first turnover is $k_2 = 1.1$ min^{-1} and the rate of subsequent turnovers is $k = 0.67$ min^{-1}. **B:** Same data as in A with only a single time point taken in the first turnover, resulting in a k_{obs} of 0.74 min^{-1}.

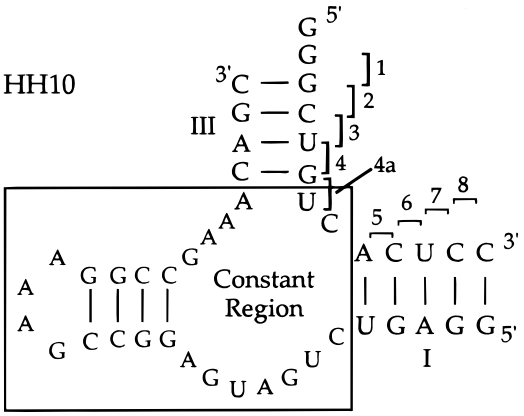
uct falling off first. The rate constants for association of each product can also be determined directly by a pulse-quench native gel analysis (Hertel et al., 1994). Although, there are not many examples in the literature, the rate constants for product association measured to date are on the order of 10^7–10^8 M^{-1} min^{-1}, similar to rates of helix association (Fedor & Uhlenbeck, 1992; Hertel et al., 1994).

It is fairly straightforward to predict whether the dissociation of a product may be rate-limiting. The affinities of ribozyme–product complexes measured to date (Fedor & Uhlenbeck, 1992; Hertel et al., 1994) correspond closely to helix stabilities calculated from empirically determined free energy parameters for RNA duplexes (Turner et al., 1988; Serra & Turner, 1995). Thus, with a calculated K_d and an estimated rate constant for product association between 10^7 and 10^8 M^{-1} min^{-1}, a product dissociation rate can be predicted. For example, a hammerhead with a calculated K_d of 2 nM for the 3′ product (P2) binding to the ribozyme would have a rate constant for product dissociation (k_4) between 0.01 and 0.1 min^{-1}. Because k_2 under standard conditions is about 1 min^{-1}, such a hammerhead would be limited by product release under multiple-turnover conditions.

PREDICTING THE RATE CONSTANTS FOR A NEW HAMMERHEAD

The rate constants for the hammerhead cleavage reaction can be understood in terms of the RNA helix-coil transition and the uniform catalytic properties of the core nucleotides. Thus, before even conducting an experiment, approximate values for each individual rate constant can be predicted for a new hammerhead having different sequences for helices I and III. For all hammerheads, k_2 is predicted to be 1 min^{-1} at pH 7.5, 10 mM $MgCl_2$, and 25 °C, and can be adjusted to other buffer conditions as described above. The value of k_{-2} is assumed to be $k_2/100$. In order to predict the rate of substrate dissociation and thereby define the kinetic regime of the new hammerhead, the substrate binding affinity must first be calculated. This procedure is outlined in Figure 8A using HH10 as an example. The values of $H°$ and $S°$ of the individual helical nearest-neighbor interactions and dangling residues that make up helix I and helix III are summed using the latest available values (Serra & Turner, 1995). This permits the total helix free energy to be calculated at the desired temperature (25 °C in the example). The constant, unfavorable free energy associated with helix initiation and the unfavorable free energy associated with the hammerhead constant region determined experimentally (K.J. Hertel, T.K. Stage-Zimmerman, G. Ammons, & O.C. Uhlenbeck, in prep.) are then added to the helix free energy to give the predicted substrate binding energy and associated K_d. By assuming a typical value of 5×10^7 M^{-1} min^{-1} for k_1, a predicted value for $k_{-1} = k_1 \cdot K_d$ is obtained. The free energies and corresponding dissociation rate constants of each product are calculated in a similar fashion (Fig. 8A). In this case, no free energy contribution for the constant region is included, but the $A_{15.1}$–$U_{16.1}$ pair is assumed to contribute to P1 stability and the presence of one product is assumed to stabilize the other by −0.9 kcal/

A



$$\Delta G^{\circ}_{E\cdot S}{}^{298} = \sum_{N=1}^{8} \Delta H^{\circ}_{NN} - (298\ K)\sum_{N=1}^{8} \Delta S^{\circ}_{NN} + \Delta G_{init} + \Delta G_{Const}$$

$$\Delta G^{\circ}_{E\cdot S}{}^{298} = -80.2\ kcal/mol - (298\ K)(-0.2073\ kcal/mol\cdot K) + 3.2\ kcal/mol + 3.1\ kcal/mol$$

$$= -12.1\ kcal/mol$$

$$K_{d(E\cdot S)} = 1/(\exp(-\Delta G^{\circ}_{E\cdot S}{}^{298}/RT)) = 1.2\ nM$$

$$\Delta G^{\circ}_{E\cdot P1}{}^{298} = \sum_{N=1}^{4a} \Delta H^{\circ}_{NN} - (298\ K)\sum_{N=1}^{4a} \Delta S^{\circ}_{NN} + \Delta G^{\circ}_{init}$$

$$\Delta G^{\circ}_{E\cdot P1}{}^{298} = -47.1\ kcal/mol - (298\ K)(-0.1229\ kcal/mol\cdot K) + 3.2\ kcal/mol = -7.27\ kcal/mol$$

$$K_{d(E\cdot P1)} = 4.4\ \mu M$$

$$\Delta G^{\circ}_{E\cdot P2}{}^{298} = \sum_{N=5}^{8} \Delta H^{\circ}_{NN} - (298\ K)\sum_{N=5}^{8} \Delta S^{\circ}_{NN} + \Delta G^{\circ}_{init}$$

$$\Delta G^{\circ}_{E\cdot P2}{}^{298} = -43.3\ kcal/mol - (298\ K)(-0.1106\ kcal/mol\cdot K) + 3.2\ kcal/mol = -7.14\ kcal/mol$$

$$K_{d(E\cdot P2)} = 5.5\ \mu M$$

B

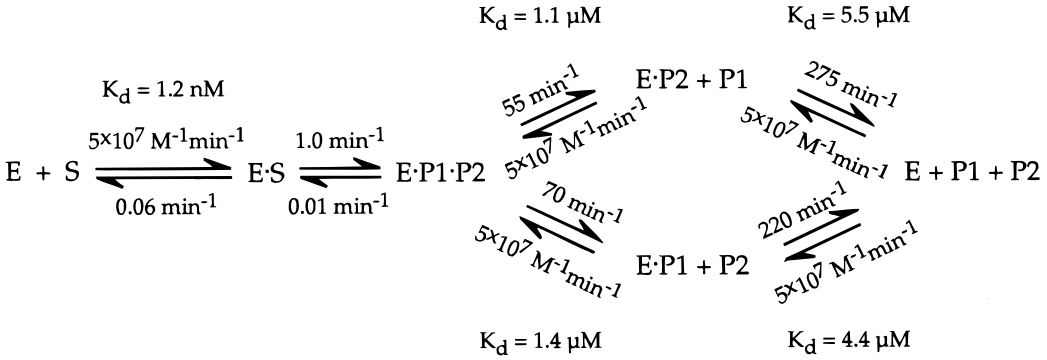


FIGURE 8. A: Schematic representation of the hammerhead ribozyme where the core nucleotides plus stem loop II comprise the "constant region" and stems I and II are the helical regions. Estimation of the substrate and product binding affinities for HH10. The free energy of substrate binding at 25 °C ($\Delta G^{\circ 298}_{E\cdot S}$) includes the enthalpy and entropy contribution for the seven nearest-neighbor interactions indicated as (2–8) and a dangling nucleotide indicated as (1) all taken from Serra and Turner (1995). Also included are the contant initiation free energy (ΔG°_{init}) and core free energy (ΔG°_{const}). The free energy of P1 binding to E ($\Delta G^{\circ 298}_{E\cdot P1}$) sums enthalpy and entropy contributions for nearest-neighbor interactions 1–4 as well as 4A and the dangling C residue. Similarly, the free energy of P2 binding to E ($\Delta G^{\circ 298}_{E\cdot P2}$) is the sum of the enthalpy and entropy contributions for nearest-neighbor interactions 5–8. **B:** Estimation of elemental rate constants for HH10.

mol (Hertel et al., 1994). The resulting set of elemental rate constants (Fig. 8B) can then be used to predict the cleavage behavior of HH10 under any initial set of ribozyme or substrate concentrations. In the case of HH10, the substrate dissociation rate constant is predicted to be much slower than k_2, placing it in the second kinetic regime. However, because all the product release steps are calculated to be faster than k_2, no burst is expected and k_{cat} should equal k_2 in a multiple-turnover cleavage reaction.

As is the case for many hammerheads, the observed cleavage properties of HH10 (Fedor & Uhlenbeck, 1990) do not entirely correspond to those predicted in Figure 8. Although a $k_{cat} = 1\ min^{-1}$ was measured in a substrate excess cleavage experiment, the fraction of substrate cleaved at saturation was only 0.4 and a very high substrate concentration was needed to reach saturation. The nonideal behavior of HH10 can arise from having a heterogeneous population of substrate that reduces the amount of active substrate available to bind the ribozyme. This situation was confirmed for HH10 by showing that the substrate formed aggregates on nondenaturing gels that did not react during the time scale of the reaction (Fedor & Uhlenbeck, 1990).

There are numerous examples in the literature where the prediction of the expected rate constants would have aided in identifying the presence of alternate conformations or helped to determine the rate-limiting step of the reaction under the conditions used. Examples listed in Table 2 were chosen because substantial cleavage data are available. In each case, the rate constants were predicted based on the sequence of the hammerhead as described above and compared with the experimental data reported. Deviations between the predicted and experimental outcomes are listed for each hammerhead and potential causes for the nonideal behavior for each hammerhead are sometimes suggested, although additional experiments would be required to identify the cause precisely. HH10 actually shows two of the most common deviations from ideal behavior: incomplete cleavage of substrate at saturation (also seen for hammerheads 1 and 2) and a very high substrate (or ribozyme) concentration required for saturation (also seen with hammerheads 3, 11–13). A third

TABLE 2. Hammerhead kinetic data from the literature.

Hammerhead	R or S excess	Deviation from estimated cleavage rate constants	Reference
#1 (R1/S13)	R	k_2(est) $\approx 4\ min^{-1}$, k_2(meas) $= 1.6\ min^{-1}$ Cleavage extent = 0.49	(Hendry et al., 1992)
	S	k_{cat}(meas) $= 0.4\ min^{-1} \ll k_2$(est) k_6(est) $\approx 0.023\ min^{-1}$ Product release partially rate-limiting	
#2 (R_{4U}, RNA)	R	k_2(est) $\geq 4\ min^{-1}$, k_2(meas) $= 0.15\ min^{-1}$ Cleavage extent = 0.59	(McCall et al., 1992)
#3 (TAT RA)	R	k_2(est) $\geq 4\ min^{-1}$, k_2(meas) $= 0.24\ min^{-1}$	(Hendry & McCall, 1995)
	S	K_M(est) = 120 nM, K_M(meas) = 1080 nM (E·S)* on pathway	
#4 (GH RA/S21)	R	k_2(est) $\geq 4\ min^{-1}$, k_2(meas) $= 0.5\ min^{-1}$	(Hendry & McCall, 1996)
#5 (Kr RA/S21)	R	k_2(est) $\geq 4\ min^{-1}$, k_2(meas) $= 0.6\ min^{-1}$	(Hendry & McCall, 1996)
#6 (HH10)	S	K_M(est) = 20 nM, K_M(meas) = 2.3 μM Cleavage extent = 0.4	(Fedor & Uhlenbeck, 1990)
#7 (2as-Rz12/2S)	R	k_2(est) $\approx 2.5\ min^{-1}$, k_{sat}(meas) $= 0.013\ min^{-1}$	(Homann et al., 1994)
#8 ($APPrbz_s/\beta App_{141}$)	R	k_2(est) $\approx 2.5\ min^{-1}$, k_{sat}(meas) $= 0.13\ min^{-1}$	(Denman, 1993)
#9 (R/HTF_{942}) (R/HTF_{452})	R	k_1(est) $\geq 1 \times 10^7\ M^{-1}\ min^{-1}$ k_1(meas) $= 0.94 \times 10^5\ M^{-1}\ min^{-1}$ k_1(meas) $= 0.11 \times 10^5\ M^{-1}\ min^{-1}$	(Jankowsky et al., 1997)
#10 (Rib#565/EDB)	S	k_2(est) $\geq 3\ min^{-1}$ k_{cat}(meas) $= 1.3\ min^{-1}$	(Paolella et al., 1992)
#11 (RS/RR)	S	K_M(est) = 150 nM, K_M(meas) = 700 nM	(Yang et al., 1990, 1992)
#12 (R_{Rh}/S_{FI})	S	K_M(est) = 150 nM, K_M(meas) = 770 nM k_1(est) $\geq 1 \times 10^7\ M^{-1}\ min^{-1}$, k_1(meas) $= 1.3 \times 10^6\ M^{-1}\ min^{-1}$	(Perkins et al., 1996)
#13 (all RNA rbz)	S	K_M(est) = 50 nM, K_M(meas) = 920 nM	(Perreault et al., 1991)
#14 ($mdrRz/S_{short}$)	S	k_2(est) $\geq 3\ min^{-1}$ k_{cat}(meas) $= 1\ min^{-1} \therefore k_{cat} \neq k_2$ k_4(est) $\approx 0.2\ min^{-1}$ Product release partially rate-limiting	(Holm et al., 1995)
#15 (rz GH/rGAG)	S	k_2(est) $> 6\ min^{-1}$, $k_{cat,burst}$ (meas) $= 0.25\ min^{-1}$ Product release partially rate-limiting.	(Taylor et al., 1992)

common observation is that the cleavage rate at saturation is much less than the value of k_2 predicted for the reaction buffer conditions. If the rate was measured with substrate excess, this can simply be explained by the fact that one of the product release steps is fully or partially rate-limiting (possible for hammerheads 1, 14, 15). This hypothesis can be tested by measuring k_2 directly in a ribozyme excess cleavage reaction or by performing a brust experiment. Another possible reason for a slow cleavage rate is that saturation was not achieved. Finally, as discussed above, a slow cleavage rate can be the result of a slow exchange from an inactive E·S conformation. Evidence for this type of behavior exists for hammerhead 3, where the pH–rate profile was not log-linear at high pH. Presumably, this observation is due to a rate-limiting conformational change, [E·S]′ to (E·S), under these conditions (Hendry & McCall, 1995).

CONCLUSIONS

The general kinetic properties of the hammerhead cleavage reaction are reassuringly simple. The substrate binding and product release steps are dominated by the well-understood RNA helix-coil transition, whereas the cleavage–ligation step is the same for virtually all hammerheads. Unfortunately, this simple view is complicated by the propensity of even quite short RNA oligonucleotides to fold into alternate structures that often significantly perturb the thermodynamics and kinetics of the cleavage reaction. These alternate structures can either be intramolecular hairpins or intermolecular aggregates, which often can be detected by other experimental methods, but sometimes cannot. Their presence is difficult to predict accurately using RNA-folding programs, presumably because noncanonical base pairs often contribute to their stability. It is likely that many of the differences among hammerhead cleavage rates observed by different laboratories can be attributed to these alternate structures. It is clear that diligent attention to correctly analyzing kinetic data is required. In this regard, the procedures to predict each elemental rate constant for a new hammerhead sequence, as outlined in this review, should be helpful not only to detect alternate conformers, but also to design hammerheads with desired kinetic properties.

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