

Measurements of weak interactions between truncated substrates and a hammerhead ribozyme by competitive kinetic analyses: implications for the design of new and efficient ribozymes with high sequence specificity

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ABSTRACT

Exploitation of ribozymes in a practical setting requires high catalytic activity and strong specificity. The hammerhead ribozyme R32 has considerable potential in this regard since it has very high catalytic activity. In this study, we have examined how R32 recognizes and cleaves a specific substrate, focusing on the mechanism behind the specificity. Comparing rates of cleavage of a substrate in a mixture that included the correct substrate and various substrates with point mutations, we found that R32 cleaved the correct substrate specifically and at a high rate. To clarify the source of this strong specificity, we quantified the weak interactions between R32 and various truncated substrates, using truncated substrates as competitive inhibitors since they were not readily cleaved during kinetic measurements of cleavage of the correct substrate, S11. We found that the strong specificity of the cleavage reaction was due to a closed form of R32 with a hairpin structure. The self-complementary structure within R32 enabled the ribozyme to discriminate between the correct substrate and a mismatched substrate. Since this hairpin motif did not increase the K_m (it did not inhibit the binding interaction) or decrease the k_{cat} (it did not decrease the cleavage rate), this kind of hairpin structure might be useful for the design of new ribozymes with strong specificity and high activity.

INTRODUCTION

Hammerhead ribozymes are small catalytic RNAs that can cleave phosphodiester linkages at specific sites in RNA substrates. It is easy to design a ribozyme that can attack a

specific target RNA and, therefore, it is anticipated that such ribozymes will be of considerable practical use (1–15). It is important, for practical applications, that hammerhead ribozymes should discriminate between matched and mismatched substrates and there have been several attempts to improve the specificity of hammerhead ribozymes (12,16–18).

Various important improvements have been achieved that have included truncation of substrate-binding arms (16,17), generation of self-complementary structures at substrate-binding sites (18) and creation of allosterically controllable ribozymes, known as maxizymes (12,13,19,20). In the first case, truncation of the substrate-binding arms enhanced the rate of dissociation of mismatched substrates from the ribozyme and, thus, mismatched substrates dissociated from the ribozyme–substrate complex before they could be cleaved. In the second case, self-complementary structures within the binding arms prevented mismatched substrates from binding to ribozymes. However, these modifications were introduced at the cost of unwelcome changes in k_{cat} and K_m , namely the rate of cleavage and binding affinity for the correct substrate(s) (16–21).

A 32mer hammerhead ribozyme, R32 (see Fig. 1A), was designed in our laboratory and its catalytic activity has been characterized in detail (4,5,22–30). R32 has high cleavage activity and the cleavage approaches completion at saturating concentrations of the ribozyme and trace levels of labeled substrate, i.e. under single-turnover conditions (4,5). Thus, populations of R32 appear to consist of fully active molecules (21–28,30). The cleavage activity of R32 is fairly high (21), with $k_{cat} > 4 \text{ min}^{-1}$ at 25 mM MgCl_2 , 37°C and pH 8.0. Since R32 cleaves a matched substrate specifically, an analysis of its mechanism of discrimination should provide us with information that will be useful for the design of ribozymes that have high cleavage activity and sequence specificity.

In this paper, we demonstrate the strong specificity of R32. We also present a detailed kinetic analysis of the weak interactions between R32 and various truncated substrates. The

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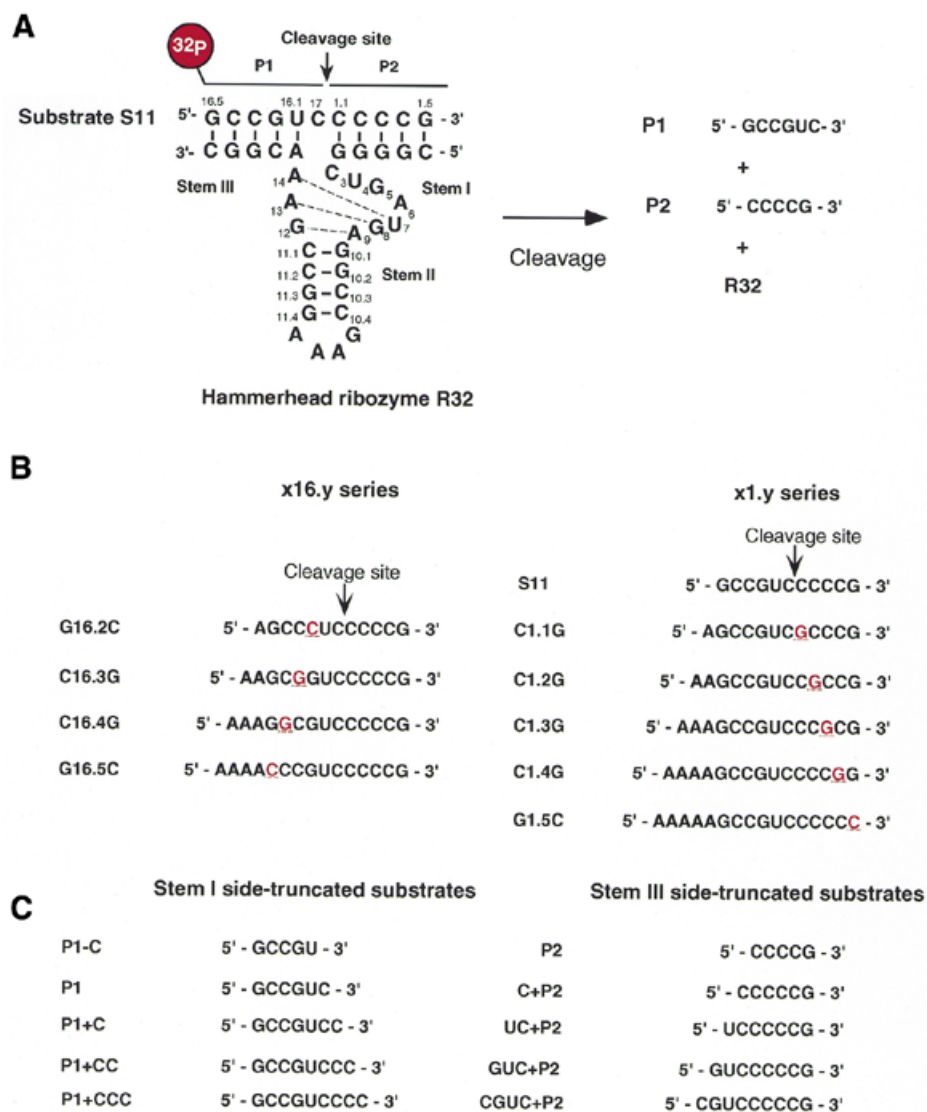


Figure 1. Secondary structure of R32 and sequences of oligonucleotides used in this study. (A) Hammerhead ribozyme R32 and its substrate S11. An arrow indicates the cleavage site. (B) Substrate S11 and the point mutants used for analysis of the specificity of R32. Each underlined red letter indicates a single nucleotide mutation introduced into the substrate. (C) The truncated substrates used for investigations of binding affinity. Each substrate corresponds to S11 truncated at its 5'- or 3'-end. P1 and P2 refer to truncation on the 3'- and 5'-side of S11, respectively.

results of our analysis suggest a new method for designing hammerhead ribozymes with high activity and strong specificity.

MATERIALS AND METHODS

Synthesis and purification of RNAs

The hammerhead ribozyme R32 and its substrate, S11, were chemically synthesized with a DNA/RNA synthesizer (model 394; PE Applied Biosystems, Foster City, CA). The newly synthesized products were deprotected and purified by standard methods, as described elsewhere (22–25,30,31). The sequences of RNA oligomers with single point mutations, designated the x16.y and x1.y series and used for studies of the specificity of R32, are listed in Figure 1B. They were purchased from Genset Oligos (Paris, France). The sequences of the stem side-truncated substrates used for determinations of K_i are listed in Figure 1C. These substrates were synthesized as described above.

Measurements of the kinetics of reactions catalyzed by the hammerhead ribozyme

We performed cleavage experiments to examine the ability of R32 to distinguish between correct and mismatched substrates. R32 and a mixture of unlabeled and 5'-³²P-labeled substrates (S11, x16.y and x1.y) were mixed in the reaction buffer without MgCl₂ before reactions were initialized. Each reaction mixture contained an equal amount of each correct and mutated substrate. All reactions were started by addition of a MgCl₂ solution and reactions were performed at 37°C in a buffer that contained 25 mM MgCl₂ and 50 mM Tris-HCl, pH 8.0. Aliquots were removed from reaction mixtures at appropriate times and mixed with stop solution (100 mM EDTA, 7 M urea, 30% glycerol, 0.1% xylene cyanol and 0.1% bromophenol blue). Products and uncleaved substrates were separated by electrophoresis in a 20% polyacrylamide/7 M urea denaturing gel and were detected with an Image Analyzer (Storm 830; Molecular Dynamics, Sunnyvale, CA).

To determine the binding affinity of each truncated substrate, we performed cleavage experiments using each-truncated substrate as an inhibitor and calculated the inhibition constant (K_i) in each case. First, we performed multiple-turnover experiments. The concentration of S11 was varied from 10 to 5000 nM depending on the truncated substrate being tested. All reactions were performed with at least a 20-fold excess of substrate over ribozyme. The concentrations of truncated substrates were varied from 10 to 5000 nM. The ribozyme, substrate, truncated substrate and a trace amount of 5'-³²P-labeled substrate were mixed together in the same reaction buffer. All reactions were started by addition of a MgCl₂ solution and reactions were performed at 37°C in a buffer that contained 25 mM MgCl₂ and 50 mM Tris-HCl, pH 8.0. Aliquots were removed from reaction mixtures at appropriate times, mixed with stop solution and analyzed as described above. Individual rate constants were obtained from initial rates of reactions. We calculated the apparent K_m value (K_m') from Eadie-Hofstee and Lineweaver-Burk plots. We confirmed that inhibition by truncated substrates was competitive (see Results). Lineweaver-Burk plots at each concentration of a specific inhibitor had the same intercept on the y-axis (32–34). In competitive inhibition, the relationship between K_m' and the concentration of inhibitor is as follows:

$$K_m' = K_m + (K_m \times [I])/K_i \quad 1$$

where K_m , K_m' , K_i and $[I]$ indicate the binding constant, the apparent binding constant, the inhibition constant and the concentration of truncated substrate, respectively. Using 1, we calculated K_i from K_m' and $[I]$ profiles.

RESULTS AND DISCUSSION

The R32 ribozyme cleaved the correct substrate significantly more rapidly than the mismatched substrates

To investigate whether the hammerhead ribozyme R32 can effectively recognize and cleave its correct substrate specifically in a mixture of correct and mutated substrates, we performed cleavage experiments with a mixture that included the correct substrate and individual mutant substrates (Fig. 1A and B). In order that all reactions were performed under identical conditions, each mutant was attached to a sequence of A residues of different length so that all mutants could be included together and could be subjected to gel separation for subsequent quantification (Fig. 1B). Thus, the reaction mixture contained four or five labeled RNAs, including the stem I side- (x1.y series) or stem III side-mismatched (x16.y series) substrates, and the concentration of each substrate was kept the same in each reaction. The substrates shown in Figure 1B have systematic single nucleotide mutations at each binding site for the hammerhead ribozyme R32; for example, G16.2C denotes G16.2 with a G→C mutation.

If R32 has the ability to cleave the correct substrate specifically, the correct substrate should be cleaved much more rapidly than mutant variants in a mixture of correct and variant substrates. As shown in Figure 2, we found that under both single- (Fig. 2B) and multiple-turnover (Fig. 2A) conditions, the correct substrate S11 was specifically cleaved. During the 1 h for which results are shown, little cleavage of substrates

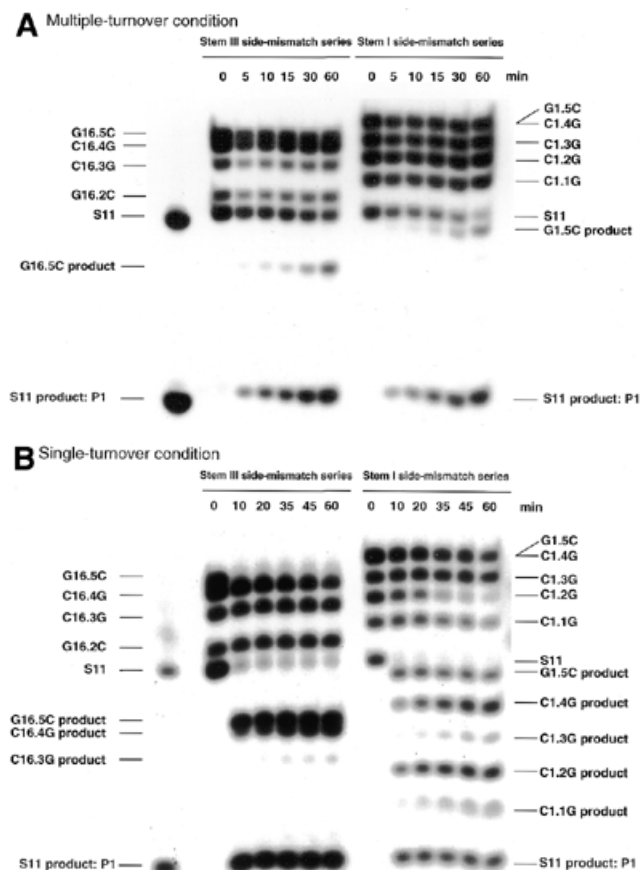


Figure 2. Autoradiographs of polyacrylamide gels showing the cleavage of each variant of S11 as a function of time. (A) Multiple-turnover experiment: 1 nM R32, 50 mM Tris-HCl, 25 mM MgCl₂ and 40 nM total substrate at 37°C and pH 8.0. (B) Single-turnover experiment: 30 nM R32, 50 mM Tris-HCl, 25 mM MgCl₂ and 20 nM total substrate at 37°C and pH 8.0. We could not distinguish between bands C1.4G and G1.5C on the gel since they were very close to each other. We exposed these mismatched substrates to alkaline hydrolysis and enzymatic hydrolysis and confirmed the assignments (data not shown).

with point mutations was detected and only very limited cleavage of mismatched substrates was detected after further incubation. In the case of G16.5C, 5'-r(AAA ACC CGU CCC CCG)-3', and G1.5C, 5'-r(AAA AAG CCG UCC CCC C)-3', in which a single mismatched nucleotide was placed at either the 5'- or the 3'-end of S11 with the remaining nine nucleotides making a continuous perfect match, significant cleavage was detected. Because of such cleavage, G16.5C and G1.5C were not used as competitors of S11 in subsequent analysis of the weak binding interactions between truncated substrates and R32.

Measurements of weak binding interactions between R32 and truncated substrates

In order to investigate the origin of the high specificity of R32 in terms of its binding affinity for its correct substrate, we performed inhibition experiments with the systematically truncated substrates shown in Figure 1C. Each substrate corresponded to S11 truncated at its 5'- or 3'-end. In our nomenclature, P1+CCC and GUC+P2, for example, denote P1 with an additional 3'-CCC and P2 with an additional 5'-GUC, respectively. We anticipated that the rates of cleavage of these

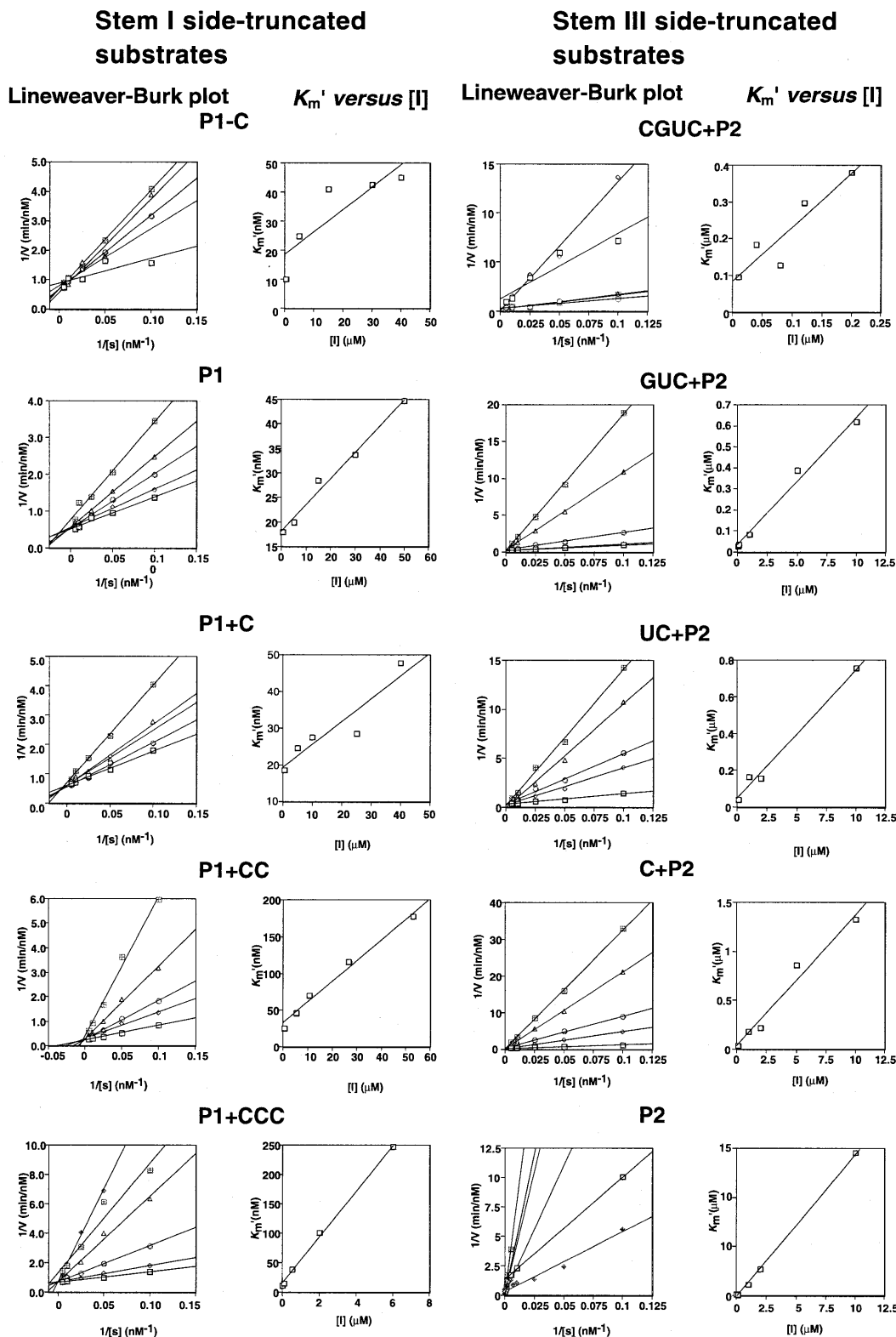


Figure 4. Results of inhibition experiments. Lineweaver–Burk plots and graphs of K_m' versus $[I]$ plots are shown. All truncated substrates were confirmed to be competitive inhibitors of S11 in the R32-catalyzed cleavage reaction from Lineweaver–Burk plots.

substrates, the shorter the truncated substrate, the more difficult it was for the altered substrates to compete with the standard substrate S11 because the truncated substrates had shorter regions that could bind to the P2 binding site. Furthermore,

the P1 binding site, which is embedded in the hairpin structure, must be opened up before the stem I side-truncated substrates can bind to R32. Thus, more energy is required for their binding to R32, as reflected in their larger K_i values.

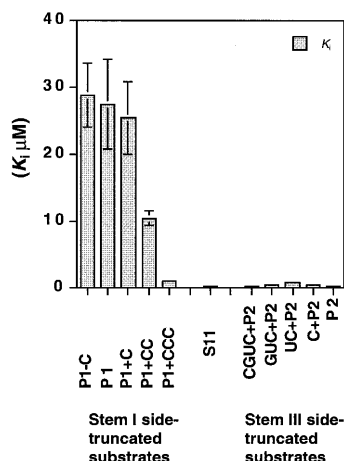


Figure 5. A summary of the results of inhibition experiments. The horizontal axis corresponds to truncated substrates, as indicated, and the vertical axis shows K_i for each truncated substrate except for S11, which is the correct substrate for R32. The value for S11 reflects K_m .

Hammerhead ribozymes have the intrinsic ability to discriminate the correct substrate from an incorrect variant. Especially, when a substrate has a single mismatch next to the cleavage site, the cleavage efficiency of hammerhead ribozymes is significantly reduced (36). However, it is possible for an incorrect substrate with some mismatches to be cleaved when binding between the ribozyme and the incorrect substrate is strong enough or when the substrate dissociation rate is low enough. Therefore, binding affinity is one of the major parameters that determine specificity.

R32 appears to be an ideal ribozyme. Its cleavage rate is one of the highest among various known hammerhead ribozymes (4,5,21). In solution, the closed hairpin structure is in equilibrium with the open form and the addition of substrate shifts the equilibrium to the open form, with subsequent formation of the R32–substrate complex. Because of this rapid equilibrium, all molecules of R32 remain in an active conformation.

Figure 7 shows a hypothetical energy diagram for the binding of R32 to its substrate. Formation of the intramolecular hairpin structure stabilizes the ground state of the ribozyme itself. As a result, mismatched substrates that have formed complexes with R32 are more likely to dissociate from such complexes than substrates in complexes with the corresponding putative open complex. Earlier investigators examined the reaction either by shortening the binding arms (16) or by creating self-complementarity at each binding arm (18). In such cases, the relative energy level of the improved ribozyme and its substrate was reduced relative to the corresponding energy level of the authentic ribozyme and substrate, with resultant higher specificity. A similar approach is also applicable to antisense molecules (37).

In the putative case of the open form shown in Figure 7A, the free energy of free R32 and its substrate is higher than that of the ribozyme–substrate complex and also than that of the ribozyme–mismatched substrate complex. Therefore, it is possible that the ribozyme might form complexes with mismatched substrates and cleave them. In contrast, in the case of the closed form of the ribozyme (Fig. 7B), free R32 and its substrate become more stable as a result of formation of a

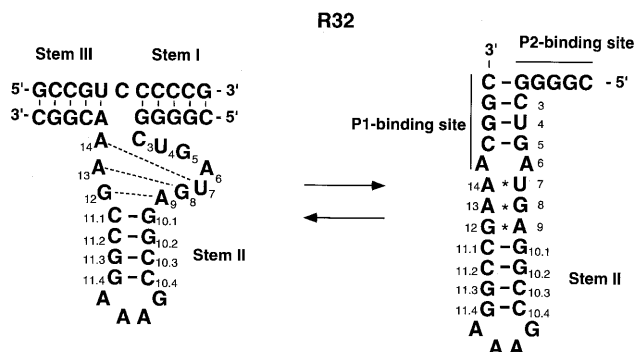


Figure 6. Open (left) and closed (right) structures of the hammerhead ribozyme R32. The closed form of R32 is a long hairpin structure, in which an additional intramolecular 4 bp duplex is formed.

hairpin structure and the free energy is reduced. Under optimal conditions, we can expect the closed form of the ribozyme to be more stable than the ribozyme–mismatched substrate complex and less stable than the ribozyme–substrate complex.

In contrast to other approaches, the advantage of the hairpin motif of R32 is the fact that, because of weak interaction within the stem region created by the P1 binding site, a relatively low K_m is retained and the rapid equilibrium between the closed and open forms of R32 in solution does not create a strong inactive form of the ribozyme. Moreover, k_{cat} is still large (21). This motif might be of limited utility because, for appropriate cleavage, the target RNA should include the 5'-YCCGUXZ-3' sequence, where Y can be any base as long as it is complementary to Z and X can be any base except G. A rapid search for such a sequence within 1128 nt of the coding region of the mRNA for actin enabled us to identify two potential cleavage sites. Therefore, it may not be unrealistic to attempt to use such a motif for the highly specific and rapid cleavage of a target molecule.

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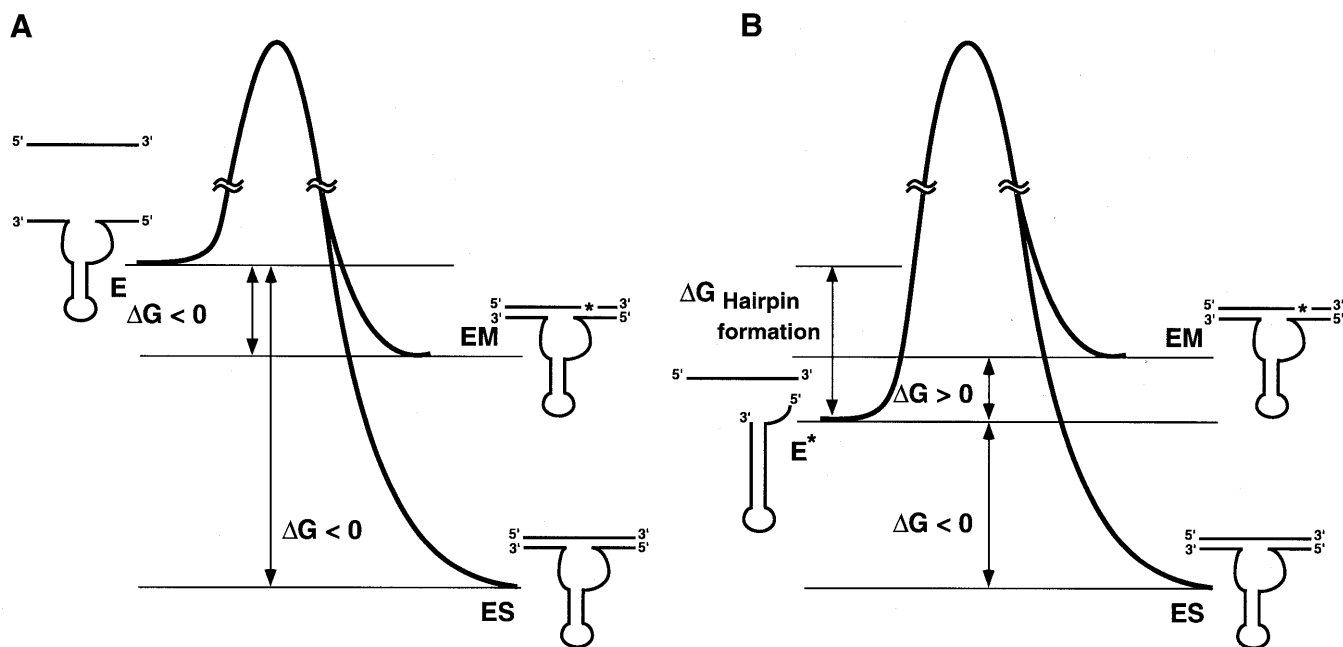


Figure 7. Hypothetical energy diagrams for the hammerhead ribozyme R32 in the open (A) and closed (B) forms. E, E*, ES and EM indicate open form R32, closed form R32, R32-substrate complex and R32-mismatched substrate complex, respectively.

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