Enhancement of ribozyme catalytic activity by a contiguous oligodeoxynucleotide (facilitator) and by 2'-O-methylation

John Goodchild*

Worcester Foundation for Experimental Biology, 222 Maple Avenue, Shrewsbury, MA 01545, USA

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ABSTRACT

RNA catalysts (ribozymes) designed to cleave sequences unique to viral RNA's might be developed as therapeutics. For this purpose, they would require high catalytic efficiency and resistance to nucleases. Reported here are two approaches that can be used in combination to improve these properties. First, catalytic efficiency can be improved by oligonucleotides (facilitators) that bind to the substrate contiguously with the 3'-end of the ribozyme. Second, 2'-O-methylation of flanking sequences of the ribozyme increases catalytic activity as well as resistance to nucleases. In combination with a facilitator oligodeoxynucleotide, the cleavage rate was increased 20 fold over that of the unmodified ribozyme.

INTRODUCTION

Processing and splicing of RNA is, in some cases, self-catalyzed or catalyzed by other RNA molecules (1). While this is not well understood mechanistically, it is now possible to design ribozymes that cleave specific sequences in target RNA's by employing one of several naturally occurring catalytic motifs (2). The simplest and most commonly used is known as the hammerhead (3). Ribozymes based on this model contain a central core of 22 nucleotides of which 13 are invariant for maximum activity (4,5) and also flanking sequences that hybridize with the substrate RNA by Watson–Crick base pairing.

One application of ribozymes might be as antiviral agents. Cleavage would be at a sequence in viral RNA not occurring normally in the host cells. In support of this approach, cells transformed to express ribozymes that cleave sequences from human immunodeficiency virus (HIV-1) were found to be protected against the virus (6). In another study, ribozymes were shown to be more efficient than antisense RNA at inhibiting expression of tumor necrosis factor in human cells (7). For the exogenous use of ribozymes against viruses, maximum catalytic activity and resistance to nucleases would seem to be desirable. Both present problems in designing ribozymes for this purpose.

Catalytic activity demands high specificity and turnover in the cleavage reaction. It was suggested that the extent and type of

base pairing would affect the turnover of ribozymes (4,8,9). We previously studied the cleavage of a sequence from human immunodeficiency virus (HIV-1) RNA by various hammerhead ribozymes and found that longer sequences gave stronger binding between the ribozyme and the cleavage products and thus decreased turnover (10). Although other factors may influence the precise number of base pairs that a ribozyme needs to form with its substrate for optimum discrimination from all other RNA's in a cell, statistical considerations suggest that 11–15 are required (11). This obviously limits the achievable rate of cleavage.

Nuclease resistance of ribozymes might be increased by chemical modifications. A number of modified nucleotides have been introduced into the central region of hammerheads to probe the catalytic process. Almost all such changes reduced catalytic activity (12–21) except a few in non-conserved regions that were without effect (22).

Reported here are two approaches to improve ribozyme performance. One is the use of oligonucleotides referred to as facilitators that hybridize to the substrate RNA contiguously with the ribozyme. The other is the incorporation of 2'-O-methyl nucleosides into the flanking sequence of the ribozyme. In combination, these improve several aspects of ribozyme performance including at least a twenty fold enhancement in the rate of substrate cleavage.

MATERIALS AND METHODS

Synthesis of oligonucleotides

Substrate RNA, radiolabelled internally using [α - 32 P]UTP, was prepared as described previously (10) using T7 RNA polymerase and chemically synthesized single stranded templates with a double stranded promoter (23). Oligodeoxynucleotides were synthesized as described previously using standard automated phosphoramidite procedures then purified by polyacrylamide gel electrophoresis (10). Ribozymes were synthesized using the automated solid-support phosphoramidite method with commercial 2'-O-silyl nucleoside phosphoramidites (24). Chimeric RNA's were prepared by incorporating 2'-O-methyl (25) or 2'-deoxynucleoside phosphoramidites during the

* Present address: Hybridon Inc., One Innovation Drive, Worcester, MA 01605, USA

synthesis. After deblocking, products were purified by polyacrylamide gel electrophoresis in the same way as substrate RNA. Unmodified ribozyme prepared by either the chemical or enzymatic routes had the same catalytic activity (data not shown).

Concentrations of radiolabeled substrate were obtained using the specific activity of the RNA determined from that of the [α - 32 P]UTP used in the preparation. Concentrations of unlabelled RNA and the DNA/RNA chimera were obtained spectroscopically from the absorption at 260nm. Extinction coefficients at this wavelength were determined from the sum of the coefficients of the component nucleotides allowing for the hypochromicity of the RNA observed when a sample was digested to completion using snake venom phosphodiesterase and bacterial alkaline phosphatase. Extinction coefficients used to determine concentrations of oligodeoxynucleotides and 2'-O-methylated RNA were computed using the program Oligsol (26).

Cleavage reactions

A solution (45 μ L) containing ribozyme, substrate (20 equivalents relative to ribozyme) and facilitator where appropriate (30 equivalents relative to ribozyme) in 50 mM Tris.HCl (pH 7.4) was brought to 37°C. Concentrations of RNA used in different experiments are given in the figure legends. Reactions were initiated by the addition of 200 mM MgCl₂ (5 μ L). Aliquots of 5 μ L were added to 15 μ L of saturated urea:200mM EDTA (1:1 vol/vol) and put on dry ice. An equal volume of 95% formamide was added and samples were denatured by heating at 95°C for 3 minutes, analyzed by electrophoresis on 15% polyacrylamide gel containing 7M urea then autoradiographed. Products and starting material from each time point were quantitated by scintillation counting of slices cut from the gels. Results were plotted using Sigmaplot version 3.10 (Jandell Scientific).

In one case, excess ribozyme (2.2 equivalent relative to substrate) was used. The ribozyme, substrate and facilitator where appropriate (2.6 equivalents relative to substrate) were first heated together in buffer at 95°C for 3 min and cooled slowly to 37°C before initiating reaction with MgCl₂ as before.

Stability of ribozymes in fetal bovine serum

Ribozymes were 5'-terminally labelled using T4 polynucleotide kinase and [γ - 32 P]ATP. About 2 pmol of RNA with an activity of 3.7×10^5 cpm were incubated at 37°C in 20 μ L of Dulbecco's modified Eagle medium containing 1% fetal bovine serum. Samples of 5 μ L were quenched in 15 μ L of saturated urea:200 mM EDTA (1:1 vol/vol) and chilled on dry ice then analyzed by polyacrylamide gel electrophoresis and autoradiography.

RESULTS

Sequences of the ribozyme R, substrates S1, S2, and facilitators F1–5, are shown in Figure 1. The sequence of the RNA substrate was taken from a site in HIV-1 RNA that we studied previously (10). Cleavage reactions were performed using 20 equivalents of the substrate and 30 of the facilitator relative to the ribozyme.

Cleavage of S1 proceeded faster when F1 was added to the reaction (Figure 2). This effect was inversely related to the length of the facilitator (Figure 3). While F1 with 13 nucleotides gave greatest enhancement of rate, F2 with 10 nucleotides was somewhat less effective and F3 with 6 nucleotides gave only a small enhancement. A control oligodeoxynucleotide that was not complementary to the substrate had no effect on the rate (data not shown). F1 was more effective than oligoribonucleotide, F4, with the same sequence (Figure 4).

To investigate whether the primary influence of the facilitator was on the secondary structure or conformation of the substrate or else more directly on the action of the ribozyme, two experiments were performed.

First, a gap was left between the ribozyme and the facilitator. Figure 5 shows that decamer F2, which abuts the ribozyme directly, was more effective than decamer F5, which binds to the substrate three bases away from the ribozyme.

Second, a comparison was made with cleavage of the shortened substrate, S2, lacking the facilitator binding site. On steric

Figure 1. Sequences of substrate (S1, S2) and ribozyme (R) RNA's and facilitators (F1–F5). F1–F3 and F5 are oligodeoxynucleotides and F4 is an oligoribonucleotide with the same sequence as F1. Apart from five purines at the 5'-end, the sequence of S is from HIV-1 RNA and the cleavage site indicated by the arrow corresponds to position 160 (27). The unmodified ribozyme was reported previously (10). Positions of 2'-O-methyl nucleotides (when present) are indicated by boxes.

grounds, a shorter substrate should be cleaved more rapidly as the binding site will be more accessible within the random coil and, in the absence of a facilitator, this was found to be the case (data not shown). However, in the presence of F1, the longer substrate was cleaved twice as fast as the shorter (Figure 6). This shows that the facilitator actively promoted the cleavage reaction apart from any effect on the conformation of the substrate.

Facilitators might influence cleavage at either the physical steps of association and dissociation of the complex or at the chemical step of ester exchange. To distinguish between these, reactions were performed using excess ribozyme so that cleavage was independent of the rates of formation or dissociation of the complexes. Under conditions given in the Materials and Methods section, the presence of the facilitator did not change the rate of cleavage (data not shown) and so did not affect the rate limiting step which is expected here to be the chemical reaction itself. This indicates that the facilitator promotes a physical step in the process.

Figure 2. Cleavage of radiolabelled substrate S1 by ribozyme with or without facilitator F1 to give products P1 and P2 containing 20 and 13 nucleotides respectively. Reactions were performed as described in Materials and Methods using 20 equivalents of substrate and 30 of facilitator relative to the ribozyme. The starting concentration of substrate was $2.7 \mu\text{M}$. Products were separated by polyacrylamide gel electrophoresis and autoradiographed. Times of reaction in minutes are given above the lanes. Panel A: without F1. Panel B: with F1.

Figure 3. Comparisons of cleavages using facilitators F1–F3. Each reaction contained 20 equivalents of substrate S1 and 30 of facilitator relative to the ribozyme with a substrate starting concentration of $2.7 \mu\text{M}$. Half-lives of the substrate were 10 min (without facilitator), 6.9 min (with F3), 1.9 min (with F2) and 1.3 min (with F1).

To further illustrate the role of the facilitator, a chimeric ribozyme was prepared with the deoxyoligonucleotide F1 covalently joined to the 3'-end of R. The single internucleoside phosphate linkage between R and F1 drastically slowed the cleavage reaction from that where R and F1 were separate (Figure 7).

Factors that might limit the activity of ribozymes in cells include the extent of cellular uptake and rate of degradation by nucleases. As resistance of RNA to ribonucleases can be increased by 2'-O-methylation (25,28), a ribozyme containing this modification was prepared (Figure 1). Methylation was confined to the flanking sequences for two reasons. Firstly, with oligodeoxynucleotides, modification of terminal nucleotides was sufficient for protection against exonucleases (29). Secondly, as stated earlier, the modifications to the central region of the hammerhead commonly reduce its catalytic potency.

Figure 4. Comparisons of cleavages using deoxy (F1) and ribo (F4) facilitators and a control with no facilitator. Each reaction contained 20 equivalents of substrate S1 and 30 of facilitator (where indicated) relative to the ribozyme with a substrate starting concentration of $1.6 \mu\text{M}$. Data points later than 60 minutes were obtained for the control but have not been included. Half-lives of the substrate were 40 min (without facilitator), 4.9 min (with F1) and 12.3 min (with F4).

Figure 5. Comparisons of cleavages using a facilitator that abuts the ribozyme (F2) or that is separated by a gap of three bases (F5). Each reaction contained 20 equivalents of substrate S1 and 30 of facilitator relative to the ribozyme with a substrate starting concentration of $2.7 \mu\text{M}$. In another experiment starting with $1.6 \mu\text{M}$ substrate, the half-lives of the substrate were 2.8 min with F2 and 6.5 min with F5.

Figure 6. Comparison of cleavage of substrates S1 and S2 that either can or cannot hybridize with F1 respectively. Each reaction contained 20 equivalents of substrate and 30 of facilitator relative to the ribozyme with a substrate starting concentration of 0.9 μ M. The half-lives were 2.25 min for S1 and 4.63 min for S2.

Figure 7. Comparison of cleavage of S1 by R with or without F1 as described in Materials and Methods and by a chimeric ribozyme with F1 covalently linked to the 3'-end of R. Each reaction contained 20 equivalents of S1 relative to the ribozyme with a substrate starting concentration of 1.6 μ M. Only the first 30 minutes of longer time courses are shown here.

Methylation in the flanking sequences reduced the rate of degradation of the ribozyme about four fold in medium containing 1% fetal bovine serum at 37°C (Figure 8). The pattern of products suggests that degradation was by endonucleases acting on the unmodified internal sequence.

Unexpectedly, methylation of the ribozyme also increased catalytic activity. The combination of methylated ribozyme and facilitator F1 was even more active and reduced the half-life of the substrate 20-fold (Figure 9).

DISCUSSION

Several reports suggest that antisense oligonucleotides complementary to contiguous sequences on a target RNA may act cooperatively (30–32). Possibly the helix formed by the first oligonucleotide assists hybridization of the second by stacking. In the present report, it is shown that the rate of cleavage of substrate RNA by a ribozyme was enhanced by an oligonucleotide hybridizing immediately adjacent to the ribozyme. This effect

Figure 8. Degradation of methylated and unmodified forms of the ribozyme in 1% bovine fetal serum as described in Materials and Methods. Lane 1: methylated ribozyme control. Lanes 2–5: methylated ribozyme incubated with serum for 1, 6, 15 and 30 minutes respectively. Lane 6: unmodified ribozyme control. Lanes 7–10: unmodified ribozyme incubated with serum for 1, 6, 15 and 30 minutes respectively. Bands were quantitated by scanning densitometry. From plots of the results (not shown), the half-lives of the methylated (3 min) and unmodified ribozymes (0.8 min) were determined.

Figure 9. Comparison of cleavage of substrate S1 by methylated and unmethylated ribozymes with and without facilitator F1. Each reaction contained 20 equivalents of substrate and 30 of facilitator (where indicated) relative to the ribozyme with a substrate starting concentration of 2.7 μ M. Half-lives of the substrate without F1 were 10 min with the unmethylated ribozyme and 2 min with the methylated. In the presence of F1, these times were reduced to 2 min and 0.5 min respectively.

increased with the length of the facilitator as would be expected if a stable hybrid between the facilitator and the substrate is required (Figure 3).

One hypothesis is that facilitators promoted binding of the ribozyme to the correct site on the substrate. If so, it is more likely this involved an increase in the rate of association between the ribozyme and its substrate rather than in the stability of the complex, which would be expected to reduce the turnover of the catalyst. Perhaps the helix formed by the facilitator and substrate nucleated the smaller helix formed by the ribozyme flanking sequence.

Alternatively, the facilitators may have affected the conformation of the substrate by, for example, inhibiting interactions deleterious to cleavage. These might include secondary or tertiary structures involving the substrate alone or both the substrate and the ribozyme. If so, the usefulness of facilitators might be restricted to substrates forming such structures. However, two experiments suggest that the facilitator does exert a direct effect on the cleavage process although they do not eliminate the possibility that some of the rate enhancement involved an effect on substrate conformation or secondary structure.

The first experiment involved a comparison of substrate S1 with a shorter substrate, S2, that lacked the facilitator binding sequence. Other things being equal, the cleavage site in a shorter substrate should be more accessible to the ribozyme and should react faster. This was found to be the case. However, in the presence of facilitator, the longer substrate was cleaved twice as fast as the shorter (Figure 6). This difference cannot be due to any effect on substrate conformation and must reflect a direct influence of the facilitator on the cleavage process.

Secondly, the facilitator was most effective when immediately adjacent to the ribozyme rather than separated by three bases (Figure 5). It is possible that this small shift in the facilitator binding site could have significantly changed any effect on substrate structure. More likely, it indicates an advantage in stacking the ribozyme and facilitator helices. This suggestion is strengthened by a similar observation made by other workers using an alkylating oligonucleotide whose ability to cross-link with a complementary strand was increased by an adjacent oligonucleotide. This effect was lost when the oligonucleotides were separated by a small gap (33).

It seems the facilitators did directly stimulate the cleavage process but did they influence a physical step (association or dissociation) or the chemical step? These possibilities were distinguished using excess ribozyme so that the rate of cleavage was determined solely by the chemical step. Under these conditions, the facilitator had no effect on the reaction showing that its influence was on a physical step. As there is no obvious reason why a facilitator should promote dissociation of the ribozyme following cleavage, the most likely explanation is that the facilitators increased the rate of association between the ribozyme and its substrate, possibly by nucleating helix formation.

Facilitators provide a means for increasing catalytic activity while, at the same time, increasing the number of base pairs involved in the selection of the target sequence. The advantage of having two separate molecules to perform this function is demonstrated in Figure 7 and involves the effect of the length of flanking sequence on product dissociation rate. Increasing the rate of reaction at sites adjacent to the facilitator should result in improved selectivity if the time of the reaction is limited to minimize cleavage at any unwanted sites. Also, by increasing the length of the substrate RNA involved in selection of the cleavage site, facilitators may permit the use of ribozymes with shorter flanking sequences that have even greater turnover.

It is not obvious why the ribooligonucleotide, F4, was less active than the corresponding deoxy sequence, F1, but in antiviral applications, deoxy facilitators might be of dual benefit in also directing cleavage of the viral RNA by endogenous ribonuclease H.

Probably, the activity of exogenous ribozymes *in vivo* will be limited by nucleases. As with antisense oligonucleotides, chemical modifications that increase nuclease resistance should be beneficial providing they do not reduce catalytic activity. In

mechanistic investigations of ribozymes, a number of chemical modifications have been made within the hammerhead complex. These involved replacement of phosphoryl oxygen atoms with sulfur (12–15) or of 2'-hydroxyl groups with hydrogen (16–19), fluorine (19,20), amino (20) or methoxyl functions (21). With a couple of exceptions (22), all modifications to the ribozyme strand reduced catalytic activity to varying extents, the effects being cumulative with the number of changes.

The introduction of 2'-O-methylated nucleosides to improve resistance to ribonucleases was therefore limited to the flanking sequences (Figure 1). As expected, the partially methylated ribozyme survived longer than the unmodified form in fetal bovine serum (Figure 8). Surprisingly, methylation also increased catalytic activity (Figure 9).

RNA/RNA duplexes are stabilized by introducing 2'-O-methylated nucleotides into one strand (34). While this would be expected to decrease the rate of dissociation following cleavage and so reduce turnover, there may be effects on the rate of association or cleavage that compensate to increase the overall rate of reaction. Whatever the reason, methylation of flanking sequences improved catalytic activity. By also promoting nuclease resistance, this modification appears doubly useful in the design of hammerhead ribozymes.

In combination with F1, the methylated ribozyme was even more active (Figure 9). Compared with the unmodified ribozyme alone, the mixture of methylated ribozyme and F1 reduced the half-life of the substrate 20 fold. This corresponds to a turnover of at least 20 min⁻¹. Most reported *k*_{cat} values for synthetic hammerheads under similar conditions are in the range 0.5–2.8 min⁻¹ although higher values have been reported on replacing Mg²⁺ with Mn²⁺ (20). For a different catalytic motif, the hairpin, a *k*_{cat} of 7.1 min⁻¹ has been reported (9).

Further work is necessary to establish with certainty the reasons for the effects reported here and how generally they may be applied. However, both methylation of flanking sequences and the use of facilitators appear promising for the development of more potent ribozymes for research and possibly therapeutic applications.

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