Efficient Improvement of Hammerhead Ribozyme Mediated Cleavage of Long Substrates by Oligonucleotide Facilitators†

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ABSTRACT: Hammerhead ribozymes were found to be not very efficient in cleaving long RNA substrates *in trans*. Oligonucleotide facilitators, capable of affecting hammerhead ribozymes by interacting with the substrate at the termini of the ribozyme, may improve this reaction. We determined *in vitro* the effects of 18 DNA and RNA oligonucleotide facilitators on three substrates containing 39, 452, and 942 nucleotides, respectively, by estimating the facilitator influences on association between ribozyme and substrate and on the cleavage step. The effects increase with the length of the substrates. With the 39mer substrate a maximal 4-fold enhancement of the ribozyme activity could be detected, the reaction with the 942mer substrate was accelerated up to 115-fold by facilitator addition. In long, structured substrates the facilitators have the potential to preform the substrate for the ribozyme attack. Due to this preforming effect, the rate of ribozyme−substrate association was increased as well as the rate of the cleavage step. 3′-End facilitators accelerate both of these rates, largely independent on the facilitator length. The rate of the cleavage step is raised as a result of a favorable activation energy gain by these facilitators. With all substrates, the 5′-end facilitators increase the association rate between ribozyme and substrate in dependence on their length. With the 39mer substrate the 5′-end facilitators decrease the rate of the cleavage step. With the long substrates 5′-end facilitators partially increase the rate of the cleavage step due to their preforming potential with these substrates. In some examples, combinations of several 5′-end and 3′-end facilitators provide an additional improvement over single facilitators in both the association between ribozyme and substrate and the cleavage step. Results suggest that even short facilitators may be efficient effectors enhancing hammerhead ribozyme mediated cleavage of long substrates.

Hammerhead ribozymes are small catalytic RNA molecules (Symons, 1992; Bratty et al., 1993) capable of cleaving a RNA substrate *in trans* in a sequence specific way (Uhlenbeck, 1987; Haseloff & Gerlach, 1988). Since the sequence requirement for the cleavage site within the substrate is minimal (Nakamaye & Eckstein, 1994; Shimayama et al., 1995; Zoumadakis & Tabler, 1995), ribozyme activity can be directed to specific RNA's by choosing target complementary ribozyme stem sequences flanking the catalytic hammerhead motif (Haseloff & Gerlach, 1988).

This led the interest to the application of hammerhead ribozymes to inactivate RNA *in vivo*. In recent years, the potential of hammerhead ribozymes to inactivate RNA substrates has been tested successfully in many experiments [for review see Marshall et al., (1994)].

However, ribozyme-mediated inhibition of target RNA *in vivo* could not be correlated to the catalytic activity *in vitro* (Crisell et al., 1993; Homann et al., 1994; Larson et al., 1993). Beside the fact of interactions of the ribozyme with proteins within the cell (Sioud, 1994; Heidenreich et al., 1995) and the unpredictable consequences of these interactions on ribozyme activity, the length of the ribozyme stem sequences seems to be critical. Ribozymes with long stem sequences were found to be more efficient in inhibiting RNA *in vivo* than ribozymes with short stem sequences (Crisell et al., 1993). Evaluating the effect of the length of the stem sequences *in vitro* with long substrates yielded no consistent results. In some cases short ribozymes were more active (Ellis & Rogers, 1993) while in other cases long ribozymes showed higher efficiency of the association and the cleavage step at physiological temperatures (Crisell et al., 1993).

Ribozymes with long stems are expected to have lower specifity in target recognition than ribozymes with short stems (Herschlag, 1991). Additionally, the product dissociation from the ribozyme is inhibited by the stabilities of the ribozyme−product complexes (Goodchild & Kohli, 1991; Ellis & Rogers, 1993). This causes a decrease of catalytic turnover and one of the great advantages of the catalytic antisense molecules is lost.

Thus, due to their higher catalytic potential short ribozymes should be preferred in principle, particularly because the use of chemically modified ribozymes with increased nuclease resistance can be envisaged (Heidenreich et al., 1994; Paolella et al., 1992) and has recently been applied successfully *in vivo* (Lyngstadaas et al., 1995). In light of this, it should be attractive to achieve improvement in the efficiency of cleaving long substrates with ribozymes containing short stem sequences.

Some ways to address this problem have previously been tested *in vitro*. The connection of a ribozyme to an anchor sequence capable of binding apart from the target sequence increased the catalytic efficiency in comparison to a conventionally designed hammerhead ribozyme (Pachuk et al., 1994). Another promising way is the addition of proteins

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binding to nucleic acids. The nonspecific binding proteins Ncp7 and A1 were found to enhance the activity of hammerhead ribozymes to cleave short (Tsuchihashi et al., 1993; Bertrand & Rossi, 1994; Herschlag et al., 1994) and long (Müller et al., 1994; Mahieu et al., 1995) substrates. Also the glyceraldehyde-3-phosphate dehydrogenase was found to increase hammerhead activity (Sioud & Jespersen, 1996).

Improvement of the catalytic activity of hammerhead ribozymes may also be achieved by the addition of oligonucleotides capable of interacting with the substrate RNA adjacent to a terminus of the hammerhead ribozyme. These oligonucleotide facilitators were found to enhance the turnover of short substrates (Goodchild, 1992; Nesbitt & Goodchild, 1994; Jankowsky & Schwenzer, 1996) as well as the activity to cleave long substrates (Denman, 1993, 1996).

In order to understand the process of facilitator action, we previously determined the influence of different facilitators on catalytic turnover as well as on association and cleavage step of a hammerhead ribozyme with a short substrate (Jankowsky & Schwenzer, 1996).

In the present work we have extended our investigations of the facilitator action onto the ribozyme reaction with longer substrates. This kinetic analysis should provide a more detailed insight into the potential of facilitators to enhance the hammerhead ribozyme activity within the more complex system of a structured RNA substrate.

MATERIALS AND METHODS

Choice of Sequences. For our experiments we constructed a hammerhead ribozyme with seven nucleotides in every stem complementary to the human tissue factor (HTF) mRNA between the bases 1479 and 1493 of the gene (Figure 1; Mackman et al., 1989). As substrates we used three domains from the HTF mRNA, consisting of either 39, 452, or 942 nucleotides, respectively (Figure 1). All three substrates contain identical ribozyme target sequences as well as identical facilitator binding sequences.

Chemical Synthesis of RNA and DNA Oligonucleotides. Ribozyme, facilitators, and substrate S 39 were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer as described (Jankowsky & Schwenzer, 1996). All oligonucleotides were purified by denaturing PAGE[1] (20%, 7 M urea, 40 V/cm). Product bands were visualized by UV shadowing and eluted (0.5 M ammonium acetate, 1 mM EDTA) (Gait et al., 1991). Concentrations were calculated according to Borer (1976).

Substrate S 39 was 5′-labeled with [γ^{32}P]-ATP and purified as described (Jankowsky & Schwenzer, 1996).

PCR. The templates for *in vitro* transcription of S 452 and S 942 were synthesized by PCR (Chabot et al., 1992). pUC18 containing HTF cDNA was purchased from ATCC. The plasmid was digested with *Sal*I, and the 1.2 kb HTF cDNA insert band was separated on a 1% agarose gel and purified with Gene Clean II (Bio 101 Inc.).

PCR primers were synthesized on an ABI 392 DNA/RNA sythesizer using standard protocols and purified on Sephadex (NAP-5, Pharmacia). The sense primer contained the T7 promotor sequence (TAA TAC GAC TCA CTA TAG GCC GCG TCG ATC TCG CCA ACT GG), and the antisense primers determined the length of the amplification products for the templates for S 942 (GCA GTA GCT CCA ACA GTG CTT CC) and for S 452 (TCC GAG GTT TGT CTC CAG GTA), respectively.

PCR was performed in 100 μL solution containing 1 pM HTF cDNA insert, 0.4 mM primers, 0.2 mM of each dNTP, 3 mM $MgCl_2$, 2 units of *Taq* polymerase (Promega), 50 mM KCl, 10 mM Tris/HCl, and 0.1% Triton X-100. The sample was overlayed with 40 mL of mineral oil. Prior to the reaction cycles in a Biometra Personal Cycler the solution was denatured at 95 °C for 5 min. *Taq* polymerase was added during the first annealing step at 60 °C. First extension was carried out at 72 °C for 2 min, followed by 30 cycles according to the reaction scheme: 1 min at 95 °C, 1 min at 60 °C, 2 min at 72 °C. Last extension occured for 5 min at 72 °C, and finally the solutions were cooled to 0 °C. For each substrate the reaction was done 10-fold, the solutions were combined, and an aliquot was analyzed on a 1.6% agarose gel. Only one band for every substrate template appeared. To remove the *Taq* polymerase the combined solutions for every substrate were extracted with phenol/chloroform/isoamyl alcohol (25:24:1). The unincorporated dNTP's were removed on Sephadex (NAP 5, Pharmacia), and the purified templates were precipitated with ethanol.

In Vitro Transcription. For each substrate the *in vitro* transcription was performed at 37 °C in 100 μL of solution containing 50 mM Tris/HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 40 units of T7 RNA polymerase (Promega), 100 units of RNasin (Promega), and 5 μg PCR-generated template. ATP, CTP, and GTP concentrations were 500 μM each, UTP was 400 μM, and 100 μCi of [α^{32}P]-UTP (3000 Ci/mmol, DuPont) was present in order to obtain random labeled substrates.

After 60 min 5 units of RNase-free DNase (Promega) was added and incubated at 37 °C for 15 min, followed by extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and removal of the unincorporated NTP's on Sephadex. The substrates were precipitated with ethanol and analyzed on 4% denaturing PAGE (7 M urea). Only one band appeared for each substrate and no further purification was required. Incorporation was determined to be 23−25%, and recovery ranged between 25 and 30 μg of RNA substrate.

Ribozyme Reactions. Ribozyme reactions were performed in 10 μL of 50 mM Tris/HCl (pH 7.5) with 10 mM $MgCl_2$ at 37 °C unless otherwise stated. Ribozyme and substrate were denatured separately with Tris/HCl buffer at 95 °C for 2 min and immediately cooled on ice, followed by incubation at reaction temperature for 3 h. This incubation time was necessary for reproducable folding of the long substrates. Shorter incubation times resulted in irreproducible cleavage rates, whereby longer incubation times did not change the cleavage rates. After this time the facilitators were added and incubated for 15−180 min, dependent on the ratio of facilitator to substrate.

For reactions with preannealing of substrate and ribozyme the ribozyme solution was added and incubated for additional 3 h. These reactions were started by the addition of $MgCl_2$. Reactions without preannealing were started by adding the ribozyme. Cleavage rates in these reactions were not significantly altered either by addition of $MgCl_2$ immediately prior to the reaction or by incubation of $MgCl_2$ with ribozyme

[1] Abbreviations: DTT, dithiothreitol; k_1, ribozyme−substrate association rate constant; k_2, rate constant of the cleavage step; $K_{d(fac-sub)}$, dissociation constant of the facilitator−substrate complex; PAGE, polyacrylamide gel electrophoresis; TSBO, target sequence blocking oligonucleotide.

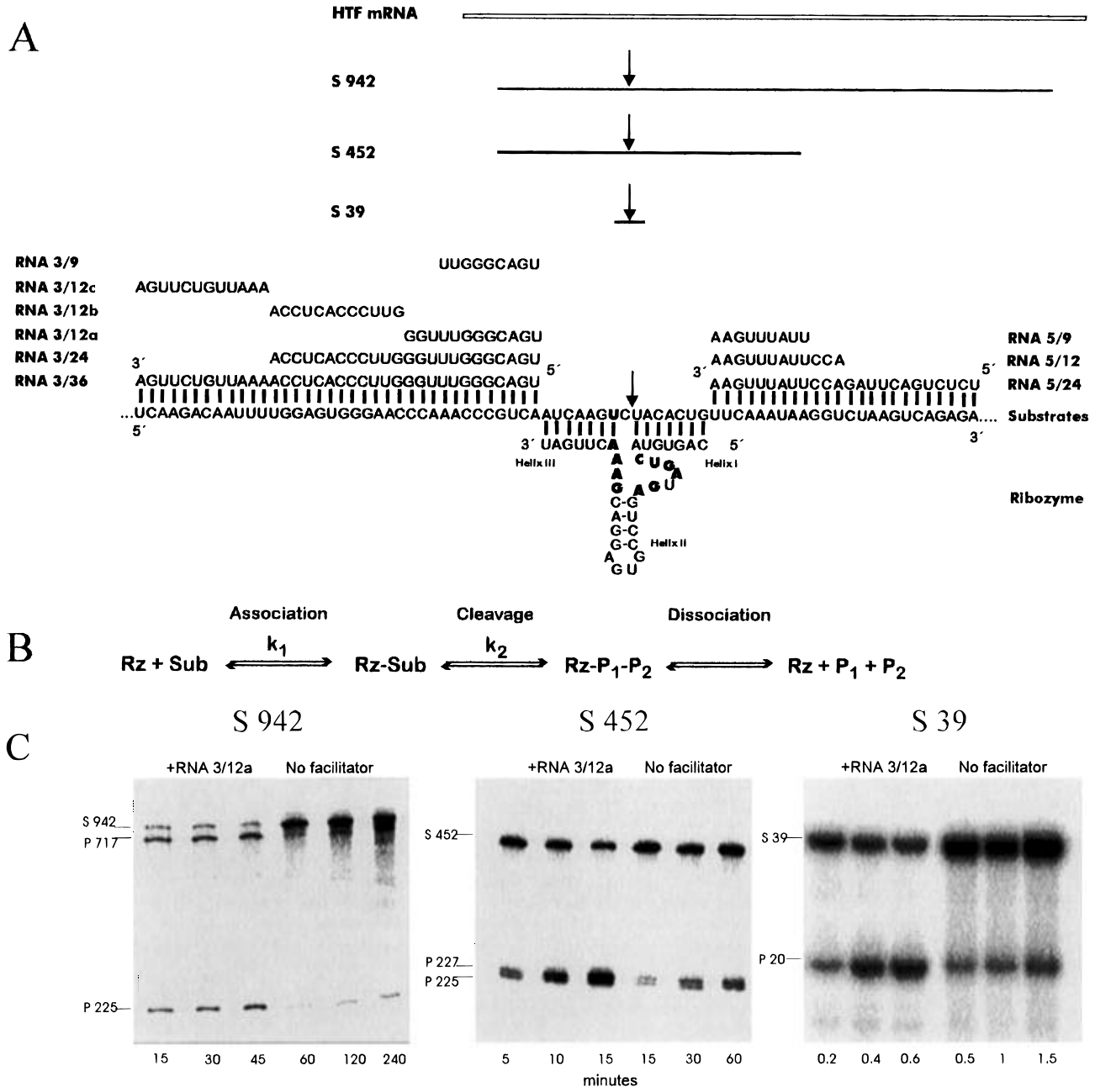

FIGURE 1: (A) Sequences of ribozyme, substrates, and facilitators. The substrates are named according to the number of nucleotides. They represent parts of different length of the HTF mRNA. These segments are indicated in the lower section of the figure, and the arrows mark the cleavage site (at nucleotide 270). The substrates S 452 (nucleotides 42−494 of the cDNA; Scarpati et al., 1987) and S 942 (nucleotides 42−984 of the cDNA) have the same 5′-terminus but differe in their 3′-terminus. The sequence of S 39 (nucleotides 250−289 of the cDNA) is contained in both of the longer substrates. Only the RNA facilitators are displayed, the DNA facilitators contain the same sequences as their RNA counterparts. Numbering of ribozyme helices is according to Hertel et al. (1992). The boldface type within the ribozyme represents the conserved nucleotides of the catalytic core. (B) Simplified kinetic scheme of the hammerhead ribozyme reaction. (C) Effect of RNA 3/12a on the cleavage of the different substrates. Reaction times are indicated. Concentrations: 100 nM ribozyme, 30 nM substrates, and 3 μM facilitator.

and substrate, respectively, for more than 60 min. This indicated that in our system Mg^{2+} does not significantly affect the structures of the long substrates.

Aliquotes of 2 μL were taken at appropriate times, added to 8 μL of ice-cold quenching buffer (8 M urea, 50 mM EDTA, 7.5% glycerine, 0.05% bromophenol blue, and 0.05% xylene cyanoblue), and separated on 4% denaturing PAGE for the longer substrates and 20% denaturing PAGE for the S 39 substrate, respectively.

Quantification was carried out with a Molecular Dynamics PhosphorImager, and nonlinear regressions were calculated using the Enzfitter software (Biosoft).

RESULTS

In reactions without facilitator the turnover decreased considerably with increasing substrate length in reactions with and without preannealing of substrate and ribozyme (Tables 1 and 2).

A quantitative comparison of the facilitator effects on the different substrates requires the determination of the rate constants of the relevant reaction steps. We determined the rate constants of the association between ribozyme and substrate as well as the rate constants of the cleavage step.

Determination of the Rate Constant of Ribozyme and Substrate Association. The rate of ribozyme and substrate

Table 1: Facilitator Effects on Association between Ribozyme and Substrate

facilitator	S 39		S 452		S 942	
	k_1[a] [$\times 10^5$ min^{-1} M^{-1}]	k_1[b] rel	k_1[a] [$\times 10^5$ min^{-1} M^{-1}]	k_1[b] rel	k_1[a] [$\times 10^5$ min^{-1} M^{-1}]	k_1[b] rel
none	43 ± 5[e]	1	0.94 ± 0.06	1	0.11 ± 0.01[c]	1
RNA 3/9	141 ± 18[d]	3.3	11.8 ± 0.9	12	5.9 ± 0.5	53
RNA 3/12a	168 ± 13[d]	4	11.3 ± 1.0	12	6.2 ± 0.4	56
RNA 3/24	−	−	7.8 ± 0.4	8	5.9 ± 0.4	53
RNA 3/36	−	−	8.6 ± 0.7	9	5.9 ± 0.6	53
RNA 3/12b	−	−	0.92 ± 0.08	1	0.10 ± 0.01[c]	1
RNA 3/12c	−	−	0.99 ± 0.09	1	0.12 ± 0.01[c]	1
RNA 3/12a+RNA 3/12b	−	−	8.9 ± 0.4	9	4.5 ± 0.6	41
RNA 3/12b+RNA 3/12c	−	−	1.02 ± 0.09	1	0.10 ± 0.01[c]	1
RNA 3/12a+RNA 3/12b+RNA 3/12c	−	−	7.0 ± 0.6	7	12.0 ± 1.0	110
RNA 3/12a+RNA 3/12c	−	−	11.1 ± 1.1	11	5.9 ± 0.5	53
RNA 5/9	60 ± 9[d]	1.4	1.32 ± 0.12	1.3	0.12 ± 0.01[c]	1
RNA 5/12	63 ± 9[d]	1.5	1.90 ± 0.21	2	0.13 ± 0.02[c]	1
RNA 5/24	−	−	4.7 ± 0.3	5	6.4 ± 0.4	58
RNA 3/12+RNA 5/12	−	−	6.3 ± 0.4	6	8.7 ± 0.9	79
RNA 3/24+RNA 5/24	−	−	6.2 ± 0.4	6	12.7 ± 1.0	115
DNA 3/9	138 ± 16[d]	3.3	1.00 ± 0.09	1	−	−
DNA 3/12a	150 ± 18[d]	3.6	11.7 ± 1.2	12	−	−
DNA 3/24	−	−	8.4 ± 0.2	9	6.3 ± 0.5	57
DNA 3/36	−	−	8.4 ± 0.6	9	5.7 ± 0.5	52
DNA 3/12a+DNA 3/12b+DNA 3/12c	−	−	7.1 ± 0.8	7	12.1 ± 1.2	110
DNA 5/9	48 ± 7[f]	1	0.90 ± 0.07	1	−	−
DNA 5/24	−	−	5.0 ± 0.6	5	6.1 ± 0.3	55
DNA 3/24+DNA 5/24	−	−	6.4 ± 0.5	6	12.4 ± 1.2	112

[a] k_1 was obtained by least-squares fits from at least three or five independent measurements of time courses of product formation (Figure 4). Each time course consists of five points. The standard errors of the curve fits were situated usually between 0.03 and 0.12. All values were obtained with 30 nM substrate, 3 μM, facilitator and 100 nM ribozyme, unless otherwise stated. Errors for k_1 represent the deviation of the average value of k_1, obtained from several determinations. [b] The relative k_1 is the quotient of k_1 with facilitator divided by the k_1 without facilitator in order to indicate the relative facilitator effect. [c] 60 nM substrate, 3000 nM facilitator, and 200 nM ribozyme. [d] 2 nM substrate, 1.5 μM facilitator, and 40 nM ribozyme. [e] This value is in very good agreement with the multiple-turnover value for $k_{cat}/K_m = 45 \times 10^5$ min^{-1} M^{-1} (Jankowsky & Schwenzer, 1996), which is also expected to reflect the association rate constant (Fersht, 1985). [f] The facilitator DNA 5/9 obviously does not form a stable duplex with the substrate S 39 at the concentrations used (Jankowsky & Schwenzer, 1996).

Table 2: Facilitator Effects on the Rate Constants of the Cleavage Step

facilitator	S 39		S 452		S 942	
	k_2[a] [min^{-1}]	k_2[b] rel	k_2[a] [min^{-1}]	k_2[b] rel	k_2[a] [min^{-1}]	k_2[b] rel
none	2.3 ± 0.3	1	0.057 ± 0.003	1	0.0185 ± 0.0020	1
RNA 3/12a	3.5 ± 0.3	1.5	0.51 ± 0.04	9	0.52 ± 0.04	28
RNA 3/24	−	−	0.46 ± 0.04	8	0.50 ± 0.04	27
RNA 3/36	−	−	0.50 ± 0.04	9	−	−
RNA 5/12	1.2 ± 0.2	0.5	0.031 ± 0.003	0.5	0.010 ± 0.001	0.5
RNA 5/24	−	−	0.10 ± 0.01	2	0.32 ± 0.03	17
RNA 3/12a+RNA 5/12	3.2 ± 0.4	1.4	0.44 ± 0.05	8	0.62 ± 0.07	33
RNA 3/24+RNA 5/24	−	−	0.42 ± 0.04	−	0.86 ± 0.06	46
DNA 3/12a	3.0 ± 0.3	1.3	−	−	−	−

[a] k_2 was determined in single-turnover experiments with preannealing. Association is not limiting under these conditions (Jankowsky & Schwenzer, 1996). Values for k_2 were determined from the slope of the plot of $-\ln(1-\text{frac[P]})$ versus time. Each plot was obtained from three (S 39) or five (S 452 and S 942) points in a time course and every time course was measured 2- or 3-fold. Concentrations were 30 nM substrates, 3 μM facilitators, and 4.5 μM ribozyme for S 452 and S 942 or 1.5 μM ribozyme for S 39. Errors for k_2 represent the deviations obtained from the calculation of the average of different determinations. Standard deviations of the fit of $-\ln(1-\text{frac[P]})$ versus time were usually in the range 0.03−0.15. [b] Relative values represent the quotient of k_2 with facilitator divided by k_2 without facilitator.

association can be monitored by the time course of product formation if the association between ribozyme and substrate becomes rate limiting for the overall reaction.

However, it has to be proven that the rate of the cleavage step is actually considerably faster than the rate of the association, i.e., whether it is possible to neglect the concentration of ribozyme−substrate complex during the reaction. We tested this by addition of oligonucleotides complementary to the ribozyme target sequences (target sequence blocking oligonucleotides, TSBO). These TSBOs are capable of inhibiting the ribozyme reaction by preventing the hammerhead association with the target. If the concentration of ribozyme−substrate complex during the reaction can be neglected, then the addition of a higher concentration of TSBO, compared with the ribozyme concentration, at an appropriate time after the start of the reaction should immediately stop the substrate turnover. This was found to be the case with all substrates (Figure 2).

This enabled us to describe the time course of reactions without preannealing and with appropriate ribozyme excess over the substrate (Figure 3) with the integrated form of the second-order reaction law for the association reaction (Young & Anderson, 1985).

$$\text{frac[P]} = R_0\{1 - \exp[(R_0 - S_0)k_1 t]\}/\{S_0 - R_0 \exp[(R_0 - S_0)k_1 t]\} \quad (1)$$

The term frac[P] is the fraction of product formed and equals frac[P] = P/S_0 (P is the product concentration). R_0 and S_0 are the initial ribozyme and substrate concentrations, respec-

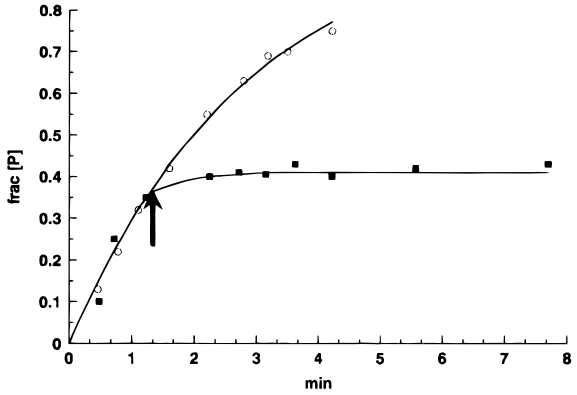

FIGURE 2: Effect of TSBO addition during a single-turnover reaction of S 39 without preannealing of substrate and ribozyme. Open circles represent the time course without addition of TSBO, and filled squares show the time course with addition of TSBO after 1.23 min (indicated by the arrow). Concentrations: 100 nM ribozyme, 30 nM substrate, 3 μM TSBO.

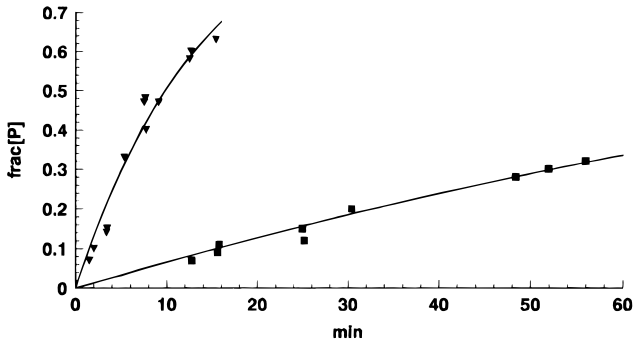

FIGURE 3: Time course of a single-turnover reaction of S 452 with RNA 3/12a (wedges) and without facilitator (squares). Concentrations: 30 nM substrate S 452, 100 nM ribozyme, 3 μM facilitator (no preannealing).

tively. The rate constant k_1 can be calculated by a least-squares fit of the time course (Figure 3).

Effect of Substrate Length on Association between Ribozyme and Substrate. The association rate k_1 decreased considerably with increasing length of the substrates. Compared with the k_1 for S 39, the association rate constant is reduced 45-fold with S 452 and 388-fold with S 942 (Table 1).

Effect of Facilitators on Association between Ribozyme and Substrate. All 3′-end facilitators increased the ribozyme−substrate association rate constant k_1 with the length of the substrates (Table 1). Whereas the 3′-end facilitators caused only a four-fold enhancement of k_1 with the shortest substrate, S 39, it was increased 10-fold with S 452 and 50-fold with S 942. The association rate improvement at all substrates was largely unaffected by the length of the 3′-end facilitators.

The 5′-end facilitators displayed different effects (Table 1). With the short substrate the 9mer and 12mer 5′-end facilitators caused small improvements of the association by enhancing k_1 by 1.5-fold. With the substrates S 452 and S 942 these facilitators did not show any effect. In contrast to the 3′-end facilitators, the effect of the 5′-end facilitators was dependent on the facilitator length. Compared with the 12mer 5′-end facilitators, a significant higher k_1 for the two longer substrates could be detected with the 24mer 5′-end facilitators. The activation effect of these facilitators was higher with S 942 than their effect on S 452.

The combined addition of 3′-end and 5′-end facilitators, tested only with the long substrates, also improved the association of ribozyme and substrate. At S 942, combinations of two 24mer 3′-end and 5′-end facilitators enhanced the k_1 by a further 2-fold, compared with the effect of a single facilitator.

No effect on the association between ribozyme and substrate could be detected with facilitators binding not directly adjacent to the ribozyme (RNA/DNA 3/12b, RNA/DNA 3/12c, Table 1).

Analogous DNA or RNA facilitators did not cause significant differences in their influence on the association with the substrates S 452 and S 942 (Table 1).

Effect of Substrate Length on the Cleavage Step. The rate constants k_2 without facilitators indicated a dependence of the cleavage step rate on the length of the substrates (Table 2); k_2 decreased with increasing length of the substrates. Compared with k_2 for S 39, the rate constant was reduced 40-fold with S 452 and 125-fold with S 942.

Effect of Facilitators on the Cleavage Step. All facilitators which bind directly adjacent to the 3'-end of the ribozyme accelerated the cleavage step (Table 2). The relative effects on k_2 increase with the length of the substrates. The length of the 3′-end facilitators did not have significant effects on the enhancement of k_2.

The effects of the 5′-facilitators on the cleavage step were length-dependent (Table 2). The facilitator RNA 5/12 reduced k_2 by the same factor at all three substrates. In contrast, the facilitator RNA 5/24 accelerated the cleavage step at the long substrates. The relative activation was not as high with S 452 as with S 942. However, the facilitator RNA 5/24 caused a significantly smaller k_2 with S 942 than the facilitator RNA 3/24 (Table 2), whereas the association rate constant k_1 was enhanced by both of these facilitators by nearly the same amount (Table 1).

With all substrates, the combined addition of 3′-end and 5′-end facilitators resulted in an activation of the cleavage step (Table 2). At S 39 and S 452 the relative effects were as high as with the 3′-end facilitator alone. At S 942 the facilitator combinations activated more than a single facilitator.

In order to obtain further information about the influence of the facilitators on the cleavage step, we estimated the facilitator effect on the transition state of the cleavage step by calculating the activation parameters from the temperature dependence of k_2 (Table 3).

The facilitator RNA 3/12a was selected to compare its effect on the three substrates. This facilitator decreased the activation entropy as well as the activation enthalpy with all three substrates, however, by a larger factor with the longer substrates than with S 39.

No significant differences in the activation parameters could be detected between analogous RNA and DNA facilitators.

No effect on the activation parameters was found for the facilitator RNA 5/12, despite its significant influence on k_2 (Tables 2 and 3).

Influence of the Facilitator−Substrate Ratio on the Facilitator Effect. The shape of the time course of product formation with increasing facilitator−substrate ratios suggests an equilibrium of facilitator−substrate complex with free substrate (Figure 4). Due to the difference of the turnover rates between reactions with and without facilitator at S 942 (Table 1) the turnover of substrate not present in the facilitator−substrate complex can be neglected. Thus, the equilibrium concentrations of facilitator−substrate complex

Table 3: Facilitator Effects on Activation Parameters for the Cleavage Reaction

substrate	facilitator	E_A[a] [kcal mol^{-1}]	$\Delta G^{\ddagger}_{(37°C)}$[b] [kcal mol^{-1}]	$\Delta H^{\ddagger}_{(37°C)}$[c] [kcal mol^{-1}]	$\Delta S^{\ddagger}_{(37°C)}$[d] [cal mol^{-1} K^{-1}]
S 39	none	26.1 ± 1.1	20.2 ± 0.1	25.5 ± 1.1	17.1 ± 1.2
	RNA 3/12a	21.9 ± 1.4	19.9 ± 0.1	21.3 ± 1.4	4.5 ± 1.5
	RNA 5/12	26.6 ± 1.7	20.5 ± 0.1	26.0 ± 1.7	17.7 ± 1.8
	DNA 3/12a	22.1 ± 1.2	20.0 ± 0.1	21.5 ± 1.2	4.8 ± 1.3
S 452	none	21.6 ± 1.7	22.5 ± 0.2	19.8 ± 1.7	-8.7 ± 1.9
	RNA 3/12a	9.2 ± 0.5	21.1 ± 0.2	8.6 ± 0.5	-40.3 ± 0.7
S 942	none	28.8 ± 1.6	23.1 ± 0.3	28.2 ± 1.6	16.5 ± 1.9
	RNA 3/12a	16.5 ± 1.0	21.1 ± 0.2	15.9 ± 1.0	−16.7 ± 1.2

[a] Activation energy E_A was determined from the product of the gas constant R with the slope of the Arrhenius plots of ln(k_2) versus 1/T. k_2 was estimated (Table 2) at 20, 25, 30, 37, 40, and 50 °C in two or three independent experiments at each temperature. Errors for E_A represent the standard deviation of the linear fit of the Arrhenius plot with the average values for k_2 at the temperatures indicated above. [b] Free activation enthalpy was calculated according to $\Delta G^{\ddagger} = -RT \ln (hk_2/k_BT)$; k_B is the Boltzmann constant, h is the Planck constant, and R is the gas constant. Errors for $\Delta G^{\ddagger}$ represent the standard deviation of the different estimations of k_2 at 37 °C. [c] Activation enthalpy was obtained according to $\Delta H^{\ddagger} = E_A - RT$. [d] Activation entropy was calculated according to $\Delta S^{\ddagger} = (\Delta H^{\ddagger} - \Delta G^{\ddagger})/T$.

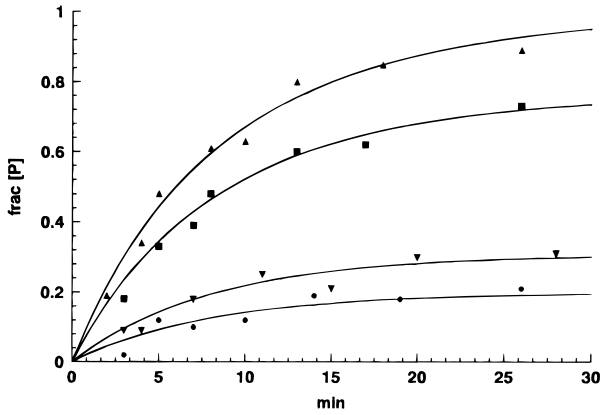

FIGURE 4: Time course of single-turnover reactions of S 942 with different facilitator−substrate ratios. The facilitator combination of RNA 3/24 and RNA 5/24 was used. Concentrations: 30 nM substrate, 100 nM ribozyme, and 30 nM (circles), 96 nM (wedges), 300 nM (squares), and 3 μM (triangles) facilitator.

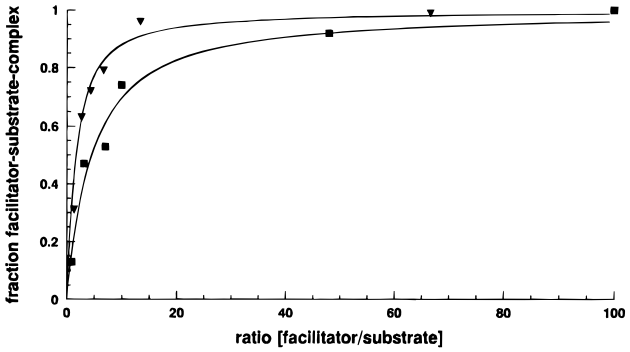

FIGURE 5: Effect of the facilitator−substrate ratio on the equilibrium concentration of facilitator−substrate complex (wedges, RNA 3/12a; squares, RNA 3/36). From this plot the dissociation constant of the facilitator−substrate complexes $K_{d(fac-sub)}$ can be determined by a least-squares fit according to the equation

$$\text{fraction[facilitator/substrate]} = \text{[facilitator−substrate complex]}/[S]_0$$

$$= 0.5(K_{d(fac-sub)}/[S]_0 + [F]_0/[S]_0 + 1) - [0.25(K_{d(fac-sub)}/[S]_0 + [F]_0/[S]_0 + 1)^2 - [F]_0/[S]_0]^{0.5}$$

which is a rearranged form of the equation for the equilibrium constant (Jankowsky & Schwenzer, 1996), where $[S]_0$ and $[F]_0$ are the initial concentration of substrate and facilitator, respectively; $\text{ratio}_{[facilitator/substrate]} = [F]_0/[S]_0$.

at increasing facilitator−substrate ratios can be calculated according to eq 1 by setting k_1 to the maximal value for the facilitator (Table 1) and calculating S_0, which under these circumstances equals the equilibrium concentration of facilitator−substrate complex. In this way, the dissociation constants of these facilitator−substrate complexes $K_{d(fac-sub)}$ can be obtained (Figure 5). We estimated for the complexes [RNA 3/24−S 942−RNA 5/24] the $K_{d(fac-sub)}$ = 121 nM, for [RNA 3/36−S 942] the $K_{d(fac-sub)}$ = 136 nM and for [RNA 3/12a−S 942] the $K_{d(fac-sub)}$ = 37 nM. For the complex [RNA 3/12a−S 942] this $K_{d(fac-sub)}$ is higher by 6 orders of magnitude than the theoretically calculated value (Turner et al., 1990).

The facilitator−substrate ratio required for the maximal facilitator effect is considerably higher for the long substrates (Figure 6). Moreover, with S 452 and S 942 longer facilitators needed higher facilitator−substrate ratios than RNA 3/12a for their maximal effect (Figure 6).

DISCUSSION

As the prerequisite for ribozyme-mediated degradation of target RNA's the structures of these RNA's have to allow first the association of ribozyme and substrate and second the proper positioning of the catalytic groups of the ribozyme in order to enable the hydrolysis of the scissile bond within the substrate. Our results suggest that, in comparison with a short substrate, both steps are inhibited within long substrates.

Effect of Substrate Length on the Association between Ribozyme and Substrate. We detected a considerably decreased rate for the association between ribozyme and substrate with the long substrates, compared with the short substrate. This is in accordance with previous observations concerning the comparison of ribozyme activity between short and long substrates with identical ribozyme target sequences (Heidenreich & Eckstein, 1992; Denman, 1993). In long structured RNA's the access of the target sequences by the ribozyme is inefficient (Heidenreich & Eckstein, 1992).

However, a portion of the long substrate has to allow the ribozyme attack as the prerequisite for the hammerhead reaction to occur. The decrease of the association rate constant for S 942 and S 452, compared with S 39, suggests that in our system only a small portion of S 942 and S 452 allows cleavage by the ribozyme. Thus, the value for k_1 should reflect conformational changes of the long substrates from conformations not suitable for ribozyme cleavage to those which can be cleaved.

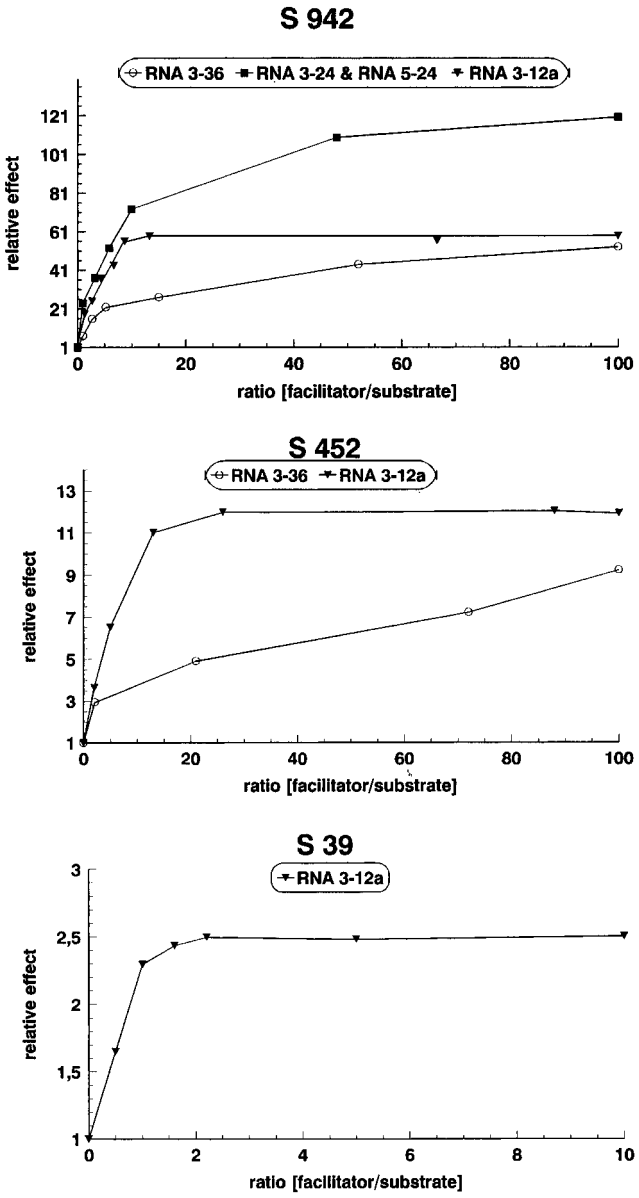

FIGURE 6: Effect of the facilitator−substrate ratio for activating effects with the different substrates. The abscissa marks the quotient of concentration of facilitator and substrate. The ordinate indicates the k_1 with facilitator divided by the k_1 without facilitator (Table 1). Concentrations: 30 nM substrates, 100 nM ribozyme.

Effect of Facilitators on the Association between Ribozyme and Substrate. It has previously been reported that the combination of short, adjacent binding oligonucleotides displays synergistic cooperativity during hybridization as antisense DNA (Maher & Dolnick, 1988) and during triple helix formation (Strobel & Dervan, 1989). This is assumed to result from enhanced stability of the hybrids due to coaxial helix stacking at both DNA and RNA (Lin et al., 1989; Walter & Turner, 1994; Walter et al., 1994). Binding of oligonucleotides adjacent to a hammerhead ribozyme has also been observed to affect the ribozyme reaction (Goodchild, 1992). At a short substrate the facilitator obviously accelerates the association between ribozyme and substrate mainly by the effect of coaxial helix stacking (Jankowsky & Schwenzer, 1996).

In contrast to their moderate effect on ribozyme−substrate association with short substrates, the facilitators cause a considerable improvement in the ribozyme−substrate association with longer substrates. Short, complementary oligonucleotides may additionally induce a conformational change in structured RNA's (LeCuyer & Crothers, 1993, 1994). Thus, due to a structural change in the environment of the ribozyme target sequence within the long substrates, driven by the facilitators, the ribozyme association is improved. This effect is large if the facilitator is capable of driving the ribozyme target sequence out of the "normal" structure of the substrate and this structural change allows an effective ribozyme association. However, the facilitator−substrate complex must have the potential to adopt a stable conformation which outweighs the facilitator mediated structural change within the substrate without involving the ribozyme target sequence in structure formation. It should be noted that even short facilitators (RNA 3/9) show a considerable effect in our system.

The different relative effects of identical facilitators with both substrates S 942 and S 452 suggest that binding of the same facilitator (or the same facilitator combination) may induce structural changes resulting in different accessebilities of the target sequences within S 452 and S 942.

The facilitator has to bind directly adjacent to the ribozyme in short substrates, otherwise no coaxial helix stacking occurs (Goodchild, 1992). With long substrates, the facilitator also has to bind directly adjacent to the ribozyme to improve ribozyme−substrate association (Table 1). The lack of an effect with facilitators binding not adjacent to the ribozyme (RNA 3/12b and RNA 3/12c) also rules out an enhancement of the ribozyme−substrate association rate due to nonspecific volume exclusion effects, which could be caused by the considerable increase of the overall RNA concentration in the experiments of facilitated cleavage versus the experiments of nonfacilitated cleavage (Table 1).

The facilitator effect on ribozyme−substrate association at long substrates, thus, results from the preforming effect and additionally from the effect of the coaxial helix stacking. The relative facilitator effects are higher with the long substrates because unfavorable structures within the substrates seems to be the main hindrance for effective ribozyme−substrate association (Dahm & Uhlenbeck, 1991). The facilitator−mediated preforming effect provides higher improvement of the ribozyme−substrate association than coaxial helix stacking.

Influence of the Facilitator−Substrate Ratio on the Facilitator Effect. A remarkable difference between the effect of the facilitators on short and long substrates could be detected in the facilitator−substrate ratios required for the maximal facilitator effect. These different ratios are the outcome of different stabilities of the complexes formed between a facilitator and the three substrates, respectively. Thus, the formation of the complexes between the facilitators and the long substrates are not as favorable as those with the short substrate. This may be the result of conformational changes within the long substrates required for the formation of the facilitator−substrate complexes. This is also evident from the lower stability of the complexes [RNA 3/36−S 942] and [RNA 3/24−S 942−RNA 5/24], compared with the complex [RNA 3/12a−S 942]. Because longer facilitators are capable to form considerable more base pairs with the substrate than shorter facilitators, longer facilitators should, theoretically, form more stable complexes (Turner et al., 1990). In contrast to this, the stability of the complex [RNA 3/36−S 942] is reduced by $\Delta\Delta G_{37°C} = 0.8$ kcal/mol, compared with the complex [RNA 3/12a−S 942], as obtained from the difference in dissociation constants of both complexes. Presumably, the energy gain by the formation of

more base pairs with the longer facilitator is required to outweigh the additional conformational strain mediated by this facilitator in comparison with the shorter facilitator.

Moreover, the facilitator−substrate ratios for RNA 3/12a and for RNA 3/36 required for the maximal facilitator effects, respectively, are very similar at both S 452 and S 942. This suggests similar stabilities of the facilitator−substrate complexes for identical facilitators with both substrates.

Although the stability of the complex [RNA 3/24−S 942−RNA 5/24] is lower than the stability of the complex [RNA 3/12a−S 942], the combination of the 24mer facilitators provide a significant higher improvement of the ribozyme−substrate association than the facilitator RNA 3/12a. Thus, the highest facilitator effect does not require the highest stability of the complex formed between substrate and facilitator.

Effect of Substrate Length on the Cleavage Step. Structures within the substrates obviously also affect the cleavage step. As the prerequisite for cleavage, the ribozyme−substrate complex must adopt a Y-shape (Amiri & Hagerman, 1994; Tuschl et al., 1994; Pley et al., 1994; Scott et al., 1995). Due to unfavorable substrate structures, the ribozyme−substrate complex may be present in a conformation which is not sufficient for the cleavage reaction. This would prevent the proper positioning of the required functional groups for the chemical step. Consequently, for the long substrates the obtained cleavage step rate constant k_2 should reflect the conformational change of the ribozyme−substrate complex into the required Y-shape. This implies a flexibility of the catalytic core of the ribozyme which has also been concluded from other observations (Herschlag et al., 1994).

Effect of the Facilitators on the Cleavage Step. No consistent observations have been published about the facilitator effect on the cleavage step. Goodchild (1992) and Nesbitt and Goodchild (1994) did not find any facilitator effect on the cleavage step. On the other hand, Denman (1993) as well as our group (Jankowsky & Schwenzer, 1996) detected such an effect.

With S 39 the 3′-end facilitators activate and the 5′-end facilitators inhibit the cleavage step. This tendency is also found with the long substrates. However, with some facilitators, these position effects are interferred by the preforming effect, which is always activating. The facilitator-mediated change of k_2 at the long substrates emphasizes that the preforming potential of the facilitators at the long substrates contributes a higher portion of the facilitator effect, compared with the influence of the facilitator position.

Further insight into the effect of the facilitators on the cleavage step can be obtained from the influence of the facilitators on the activation parameters of the cleavage step. The facilitator mediated changes of the activation parameters should indicate the conformative influence of the facilitators on the ribozyme−substrate complex within the transition state, because the facilitators hardly act directly on elementary chemical steps of the hammerhead cleavage.

The cleavage step is a monomolecular reaction which should be reflected in a positive activation entropy (Hertel & Uhlenbeck, 1995). This was found to be the case for the short substrate S 39.

However, conformational rearrangement within the substrate during the cleavage step may cause a negative activation entropy at long substrates. Additionally, the influences of metal ion binding and solvent effects on the activation entropy become more complex within longer RNA's (Li et al., 1995).

A decrease in activation entropy was detected at all substrates with the 3′-end facilitators. These facilitators may restrict the flexibility of the ribozyme−substrate complex within the transition state. This unfavorable entropy effect is outweighed by the decrease in activation enthalpy, i.e., the transition state, which is higher ordered by the facilitator, can be passed with lower energy.

The differences between the activation parameters with and without facilitator are very similar for both S 942 and S 452. Despite the different relative changes in k_2, caused by RNA 3/12a at S 942 and S 452, this facilitator might induce a conformational rearrangement with similar energetic and entropic changes in both S 942 and S 452. This fact, taken together with the very similar stabilities of the facilitator−substrate complexes of both substrates with identical facilitators and the absence of an effect by facilitators binding distant to the ribozyme, suggest, that at the long substrates only a limited domain is involved in the required conformational rearrangement for facilitator binding.

This is in accordance with investigations concerning the folding behavior of larger ribozymes. It has been reported that these ribozymes, which require a defined structure for their function, are folding in several domains. These domains adopt their structures largely in a independent manner (Zarrinkar & Williamson, 1994; Pyle & Green, 1994; Pan, 1995).

In order to obtain insight into the differences between 3′-end and 5′-end facilitator effects on k_2, the influences of more facilitators on the activation parameters were tested with S 39. The inhibitory effect of the 5′-end facilitator on the cleavage step does not significantly change the activation parameters. Although the 5′-end facilitator also binds to the substrate and should restrict the flexibility of the ribozyme−substrate complex in the transition state as well, no decrease in activation entropy is observed. However, it has been reported that the additional stability of a helix provided by coaxial helix stacking of a second, adjacently binding helix, is dependent on whether the second helix binds 3′-end or 5′-end to the first helix (Walter & Turner, 1994). This is caused by the different structures of 3′-dangling and 5′-dangling ends (Petersheim & Turner, 1983). 3′-End dangling ends (corresponding to the potential 5′-end facilitator binding sequences of the substrates, Figure 1) stack on the other strand (corresponding to the ribozyme helix I) (Petersheim & Turner, 1983) and contribute to a higher helix stabiliy than 5′-dangling ends (corresponding to the potential 3′-end facilitator binding sequences of the substrate), which do not show these stacking effects (Turner et al., 1990).

Probably, due to different structures of the facilitator binding sequences of the substrate, the binding of the 5′-end facilitator is less favorable for the transition state of the cleavage step than the dangling facilitator binding sequence without facilitator.

Conclusions. In our experiments a more than 100-fold enhancement of the ribozyme activity with a long substrate was detected with some facilitators. This increase is higher than the previously reported enhancement of the ribozyme activity by the protein Ncp7 at a substrate of comparable length (Mahieu et al., 1995).

With long substrates the facilitators have the potential to preform the substrate for ribozyme reaction. In our system even short 3′-end facilitators (9mer and 12mer) are effective.

In some cases, the combination of several facilitators further improves the ribozyme activity.

Despite a small effect on short model substrates, facilitators provide a very interesting approach to improve the activity of hammerhead ribozymes which show poor cleavage of long substrates.

For potential intracellular applications facilitators might be of higher interest for the use of short and probably modified ribozymes because the activity of a short ribozyme could be enhanced. Thus, facilitators should preferably used with exogenous delivery strategies, where ribozyme and facilitators would be co-transfected into the cells. Endogenous co-expression of ribozyme and facilitator would be possible but more complicated because both facilitator and ribozyme have to be processed correctly at their ends.

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