Fluorescence Resonance Energy Transfer Analysis of Ribozyme Kinetics Reveals the Mode of Action of a Facilitator Oligonucleotide†

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ABSTRACT: A defining characteristic of catalysts is the rate at which they can process multiple copies of substrate. In the case of synthetic hammerhead ribozymes that cleave an RNA sequence, binding of the ribozyme to the substrate and products is through base-paired duplexes. The kinetics of formation and dissociation of these duplexes can determine the turnover of the ribozyme. We have followed these processes in real time by using fluorescent labels that can interact through fluorescence resonance energy transfer (FRET). This approach has been used to identify the rate-limiting steps for a particular ribozyme and to reveal how turnover was improved by a facilitator oligonucleotide. It was found that dissociation of the ribozyme-substrate complex is faster than cleavage to products. Hence, to undergo cleavage, most substrate molecules must interact with a ribozyme more than once. In the presence of a facilitator oligonucleotide, the complex is stabilized so that cleavage is faster than dissociation. Under these circumstances, cleavage of the substrate becomes the most likely outcome following binding to the ribozyme.

Small catalytic RNA motifs such as the hammerhead or hairpin ribozyme have been used to design artificial nucleases that can cleave a selected target RNA sequence. This was first demonstrated by Uhlenbeck, who combined the catalytic core of a hammerhead motif with antisense flanking sequences in a *trans*-acting ribozyme (Uhlenbeck, 1987). The mechanism of action of these ribozymes may involve at least 12 different rate constants (Fedor & Uhlenbeck, 1992), as shown in Figure 1. Although the kinetic parameters involved in the cleavage of a target RNA by a *trans*-acting hammerhead ribozyme have been determined using electrophoretic techniques (Hertel et al., 1994), it would be preferable to follow the individual hybridization steps directly. In the work reported here, we have developed a method which allows direct monitoring of these hybridization events based on fluorescence resonance energy transfer (FRET) between fluorescein and tetramethylrhodamine fluorophores carried by the substrate and ribozyme, respectively. We have gone on to use this technique to reveal the mechanism by which a facilitator oligonucleotide increases the turnover of a small hammerhead ribozyme.

In FRET, an excited donor fluorophore transfers energy nonradiatively to an acceptor molecule. One mechanism for energy transfer involves an electrostatic interaction between the excited state donor and the ground state acceptor (Förster, 1948). A requirement is that there be overlap between the emission and absorption spectra of the donor and acceptor, respectively. The efficiency of energy transfer by this mechanism varies inversely with the sixth power of the distance between the donor and acceptor. Consequently, energy transfer increases greatly as the fluorophores are brought together.

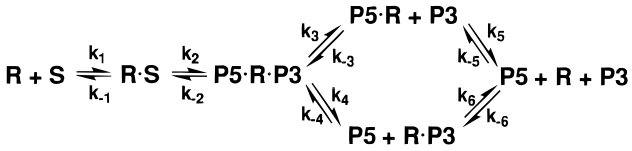

FIGURE 1: Theoretical minimal reaction pathway for cleavage of an RNA substrate S by a hammerhead ribozyme R (Fedor & Uhlenbeck, 1992).

By attachment of the donor and acceptor to complementary oligonucleotides (Clegg, 1992), FRET has been used to demonstrate hybridization (Heller & Morrison, 1985; Cardullo et al., 1988; Sixou et al., 1994; Mergny et al., 1994), to follow hybridization kinetics (Morrison & Stols, 1993; Perkins et al., 1993; Yang et al., 1994; Parkhurst & Parkhurst, 1995), and to estimate molecular distances within nucleic acids (Beardsley & Cantor, 1970; Yang & Söll, 1974; Odom et al., 1980; Robbins et al., 1981; Murchie et al., 1989; Cooper & Hagerman, 1990; Ozaki & McLaughlin, 1992; Eis & Millar, 1993; Tuschl et al., 1994).

To monitor hybridization in solution, the 3′-terminus of one oligonucleotide can be labeled with a donor and the 5′-end of its complement with an acceptor (or vice versa) (Figure 2). Prior to hybridization, energy transfer is low or nonexistent. As a result of hybridization, the fluorophores are brought together, resulting in energy transfer. Energy transfer is most directly measured in the emission spectrum where it is manifested as a reduction in the donor emission and enhancement of the acceptor emission. The kinetics of this process may be followed to determine rate constants of hybridization.

In the present work, we have employed this technique to investigate hybridization of substrates and products to a *trans*-acting hammerhead ribozyme, R_{Rh}, containing six bases in each antisense flanking sequence (Figure 3). This was

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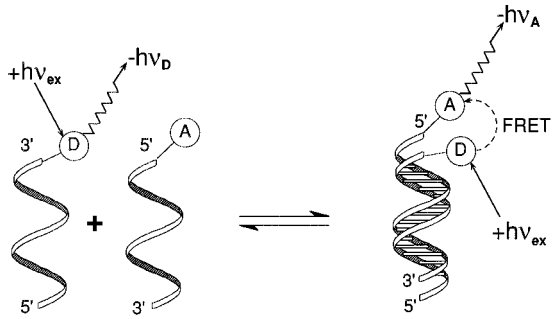

FIGURE 2: Monitoring oligonucleotide hybridization by FRET using energy donor (D)- and energy acceptor (A)-labeled oligonucleotides.

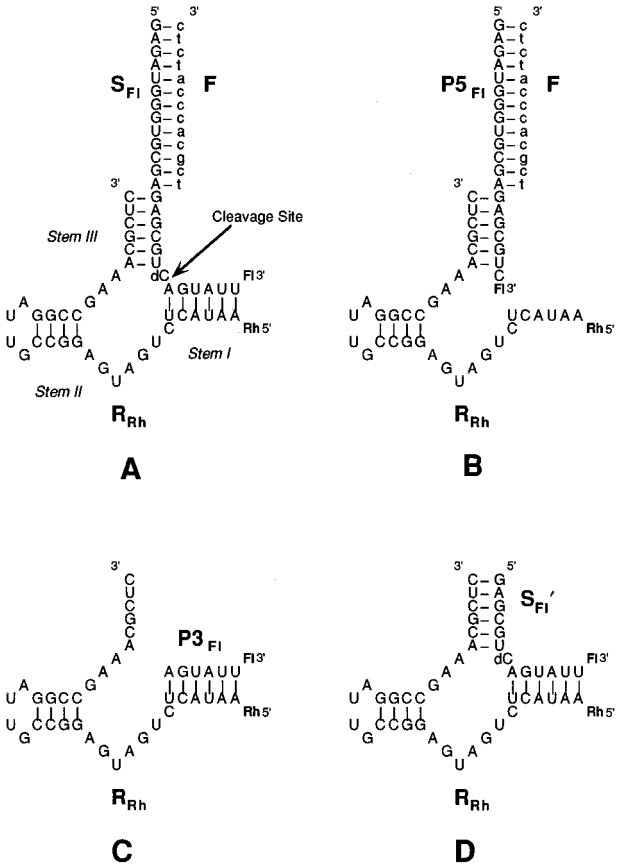

FIGURE 3: Complexes involving the tetramethylrhodamine-labeled ribozyme R_{Rh}, facilitator deoxyoligonucleotide F (shown in lowercase), and various substrates and products labeled with fluorescein. Positions of tetramethylrhodamine and fluorescein labels are indicated by Rh and Fl, respectively. (A) Complex of ribozyme with noncleavable substrate, S_{Fl}, and facilitator. S_{Fl} was rendered noncleavable by replacing cytidine with deoxycytidine at the cleavage site. (B) Complex of ribozyme with $P5_{Fl}$, the cleavage product from the 5′-end of the substrate. (C) Complex of ribozyme with $P3_{Fl}$, the cleavage product from the 3′-end of the substrate. (D) Complex of ribozyme with S_{Fl}′, a noncleavable substrate lacking the facilitator binding site. Not illustrated is S_{Fl}*, a cleavable form of S_{Fl} with cytidine at the cleavage site and labeled at the 5′-end with ^{32}P. Also, in some instances, P5 was used without a fluorescein label.

labeled on the 5′-terminus with tetramethylrhodamine. To simplify studies involving hybridization of the substrates, they were rendered noncleavable by introducing a single deoxynucleoside at the cleavage site. Two noncleavable substrates were studied: S_{Fl}, which is a 26-base RNA that contains a binding site for both the ribozyme and facilitator, and S_{Fl}′, which is a 13-base RNA that contains a binding site for the ribozyme but lacks that for the facilitator. For the investigation of cleavage kinetics, a third substrate, S_{Fl}*, having the same sequence as S_{Fl} but comprised entirely of ribonucleotides enabling cleavage to occur was synthesized. The 3′-termini of substrates S_{Fl}, S_{Fl}′, and S_{Fl}* and the cleavage products $P5_{Fl}$ and $P3_{Fl}$ were labeled with fluorescein. Fluorescein and tetramethylrhodamine are well-characterized spectroscopically with regard to Förster energy transfer with fluorescein acting as the donor (Cardullo et al., 1988; Morrison & Stols, 1993; Perkins et al., 1993; Murchie et al., 1989; Eis & Millar, 1993; Tuschl et al., 1994; Sixou et al., 1994; Yang et al., 1994).

We previously reported a way of improving hammerhead activity using facilitator oligonucleotides. These hybridize to the substrate next to the 3′-end of the ribozymes (Goodchild, 1992; Denman, 1993, 1996; Nesbitt & Goodchild, 1994; Jankowsky & Schwenzer, 1996). Facilitators were most effective when flanking sequences of the ribozyme were six nucleotides or less. We showed that facilitator oligonucleotides induce cleavage by a ribozyme with four base-flanking sequences that is otherwise inactive and they permit cleavage in 1 mM magnesium that would not normally have occurred (Nesbitt & Goodchild, 1994).

Many diseases, infectious and otherwise, are associated with overexpression or inappropriate production of some protein. Selective inactivation of the relevant mRNA, using antisense oligonucleotides or ribozymes, offers a fresh approach to therapy (Scanlon et al., 1995). This would require the development of ribozymes exhibiting properties commensurate with pharmaceutical applications. Previously, we laid out certain criteria that we thought would be helpful in achieving these requirements (Nesbitt & Goodchild, 1994). The properties of facilitators outlined above are useful in meeting a number of these criteria and could have application in ribozyme-based therapeutics.

In particular, facilitators enable the use of ribozymes with shorter flanking sequences that are easier to manufacture. They improve the turnover of these ribozymes and also their specificity by directing cleavage to the desired site. They can make the target sequence more accessible when it may be partly concealed by secondary structure (Denman, 1996). Their ability to improve cleavage in 1 mM magnesium, similar to the concentration in cells, rather than the 10 mM or more that hammerheads prefer, would seem particularly useful for achieving activity *in vivo*.

Certain proteins and peptides also have the ability to facilitate ribozyme reactions, and it has been suggested that endogenous proteins might serve this purpose in cells (Bertrand & Rossi, 1994; Heidenreich et al., 1995; Jeng et al., 1996; Mahieu et al., 1995; Müller et al., 1994; Olive et al., 1995; Sioud & Jespersen, 1996; Tsuchihashi et al., 1993). For exogenous application with ribozymes, oligonucleotide facilitators have the attraction of being chemically similar to ribozymes for purposes of pharmacokinetics, cell uptake, and intracellular partitioning.

In our previous work, we suggested that cooperative binding between the facilitator and ribozyme stabilized the binding of the ribozymes to the substrate (Nesbitt & Goodchild, 1994). However, the quantitative effect of facilitators on steps in the pathway in Figure 1 was not determined. In this paper, we report the use of FRET as a new way to determine the rate constants for the constituent steps in the hammerhead ribozyme cleavage pathway (Figure 1). We apply this method to reveal a mechanism by which facilitator oligonucleotides improve the catalytic efficiency of hammerhead ribozymes.

MATERIALS AND METHODS

Synthesis of Oligonucleotides. Oligonucleotides were synthesized on a 1 μmol scale using a Gene Assembler Plus synthesizer (Pharmacia, Uppsala, Sweden). Phosphoramidites were obtained from Perseptive Biosystems (Framingham, MA) and Glen Research (Sterling, VA) and solid supports from Glen Research. Phosphoramidites and CPG[1] supports for RNA synthesis were protected with 2′-*O*-TBDMS and PAC (on A and G) or TAC (on C) (Usman et al., 1987; Sinha et al., 1993). Capping was performed using *tert*-butylphenoxyacetic anhydride (Perseptive Biosystems).

For attachment of tetramethylrhodamine at the 5′-end of R_{Rh}, a thymidine residue carrying an amino linker on the base was incorporated (Amino-Modifier C2 dT phosphoramidite from Glen Research). For attachment of fluorescein at the 3′-end of S_{Fl}, S_{Fl}′, S_{Fl}*, $P3_{Fl}$, and $P5_{Fl}$, an amino function was introduced using an appropriately derivatized support (3′-Amino-Modifier C7 CPG support from Glen Research).

The oligonucleotides were cleaved from their supports, and the bases were deprotected by treatment with neat ethanolamine (Polushin et al., 1994). The CPG-bound material was incubated in 300 μL of ethanolamine at 70 °C for 35 min in a 1.5 mL screwcap polypropylene tube and then chilled on ice. The supernatant was removed and the support rinsed with water. The combined supernatants were divided into two equal aliquots that were evaporated overnight to an amber-colored, viscous residue. Each aliquot was dissolved in 400 μL of 1.0 M TBAF in THF (Aldrich, Milwaukee, WI) and kept for 17 h at room temperature in the dark. The mixture was chilled on ice and the reaction quenched by the addition of 400 μL of 50 mM Tris-HCl (pH 8.4) followed by 800 μL of 95% formamide with 50 mM EDTA and Orange G tracking dye. The mixture was heated to 92 °C for 3 min, chilled on ice, and purified by electrophoresis in a 15% polyacrylamide gel containing 7 M urea at pH 8.5. Product was extracted using the crush and soak technique in 0.5 M ammonium acetate (Sambrook et al., 1989). The aqueous solution was concentrated by repeated extraction with *n*-butanol and brought to 0.3 M in sodium chloride and the product precipitated by addition of 3 volumes of ethanol.

Attachment of Fluorophores. Oligonucleotides with terminal amino linker groups were labeled with the mixed isomeric *N*-hydroxysuccinimide esters of 5(6)-carboxyfluorescein and 5(6)-carboxytetramethylrhodamine (Molecular Probes, Eugene, OR) (Sinha & Striepeke, 1991). In a typical reaction, 28 nmol of oligonucleotide was dissolved in 180 μL of 0.4 M $NaHCO_3/Na_2CO_3$ (pH 9.0)/*N*,*N*-dimethylformamide/water (3:2:1 v/v/v). This solution was diluted with an equal volume of water, and 1.5 mg of active ester of the fluorophore was added. The mixture was kept at room temperature in the dark for 17 h with gentle shaking and then diluted to 6 mL with water. Most of the excess dye was removed by extraction of the aqueous solution with *n*-butanol followed by ethanol precipitation of the oligonucleotide as described above. The crude product was further purified by denaturing 15% polyacrylamide electrophoresis as described previously. Labeling was confirmed to be approximately stoichiometric from the UV−visible absorbance spectrum.

[1] Abbreviations: CPG, controlled pore glass; PAC, phenoxyacetyl; TAC, *tert*-butylphenoxyacetyl; TBDMS, *tert*-butyldimethylsilyl; TBAF, *tert*-butylammonium fluoride; THF, tetrahydrofuran; PAGE, polyacrylamide gel electrophoresis.

Table 1: Effect of Terminal Fluorescent Markers on the Free Energy of Hybridization of a 10-Base Pair DNA Duplex[a]

fluorophore	$\Delta G°_{37}$ (kcal mol^{-1})[b]	$\Delta\Delta G°_{37}$ due to fluorophore (kcal mol^{-1})
none	−11.0	not applicable
tetramethylrhodamine	−11.7	−0.7
fluorescein	−12.1	−1.1
both	−12.9	−1.9

[a] Free energies were obtained from melting curves as described in Materials and Methods. [b] Estimated error = ±0.1 kcal mol^{-1}.

^{32}P Labeling of Oligonucleotides. A 1 μL aliquot of a 10 μM solution of the oligonucleotide was combined with 5.0 μL of [γ-^{32}P]ATP (10 μCi/μL; Amersham Life Science Inc., Arlington Heights, IL), 1 μL of 10 × kinase buffer (Pharmacia Biotech, Piscataway, NJ), 0.5 μL of RNasin (33 units/μL, Promega, Madison, WI), 1 μL of polynucleotide kinase (6100 units/μL, Pharmacia Biotech), and 1.5 μL of water. The solution was incubated at 37 °C. After 1 h, 10 μL of 90% formamide containing 10 mM EDTA and 10 mM Orange G dye was added. The oligonucleotide was purified by denaturing PAGE and, following autoradiography, was isolated by crush and soak as described above.

Contribution of Fluorophores to the Free Energy of Hybridization. The free energy contributions ($\Delta\Delta G°_{37}$) of fluorescein and tetramethylrhodamine conjugates to the stability of oligonucleotide duplexes were determined from the melting curves of 10-bp DNA duplexes shown below that contain the sequence of stem I and part of stem III with and without these fluorophores.

5′-d(GCGTAGTAAT)-(Fl)

3′-d(CGCATCATTA)-(Rh)

Thermal denaturation experiments were performed on a Varian Cary 1E spectrophotometer equipped with a Peltier-controlled 6 × 6 multicell sample block on an automated sample transporter. Samples were prepared in 50 mM Tris-HCl (pH 7.3) with 20 mM $MgCl_2$ in a total volume of 1.5 mL with the concentration of each strand being 6.67×10^{-7} M. Samples were heated at 92 °C for 3 min in 1.6 mL Eppendorf tubes in an aluminum heat block. The block was allowed to come slowly to room temperature. Samples were transferred to self-masking semimicro cuvettes and sealed with a Teflon stopper. Samples were heated at a rate of 0.5 °C/min from 10 to 90 °C.

Free energy values for the dissociation of the hybrids were determined by fitting the melting curves using a two-state model (Petersheim & Turner, 1983). From the differences due to the fluorophores, their contribution to the free energy of hybridization determined as $\Delta\Delta G°_{37}$ (Table 1).

Fluorescence Spectroscopy. Fluorescence spectra (Figure 4) were obtained on an AlphaScan II fluorescence spectrophotometer (PTI, Brunswick, NJ) equipped with a 75 W high-pressure xenon lamp and a water-heated and -cooled aluminum cell block. The temperature of the cell block was controlled using a VWR 1586 water-circulating bath (VWR, West Chester, PA). Samples were placed in a 12.5 × 12.5 × 49.5 mm fluorescence cuvette. A sample volume of 3 mL was used in all cases. Excitation and emission slits were set for a spectral bandwidth of 1 nm. Emission data points were collected at 1 nm intervals with a 0.5 s signal-averaging time.

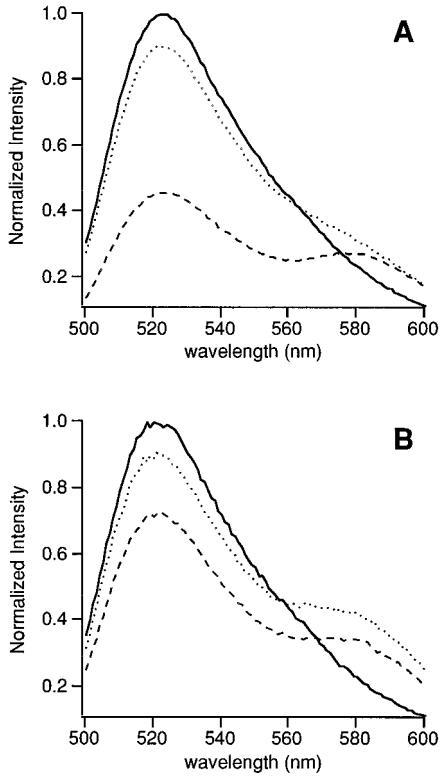

FIGURE 4: (A) Emission spectra of S_{Fl} before (—) and after addition of R_{Rh} in the absence (···) and presence (- - -) of the oligonucleotide facilitator. (B) Emission spectra of $P5_{Fl}$ before (—) and after addition of R_{Rh} in the absence (···) and presence (- - -) of the oligonucleotide facilitator.

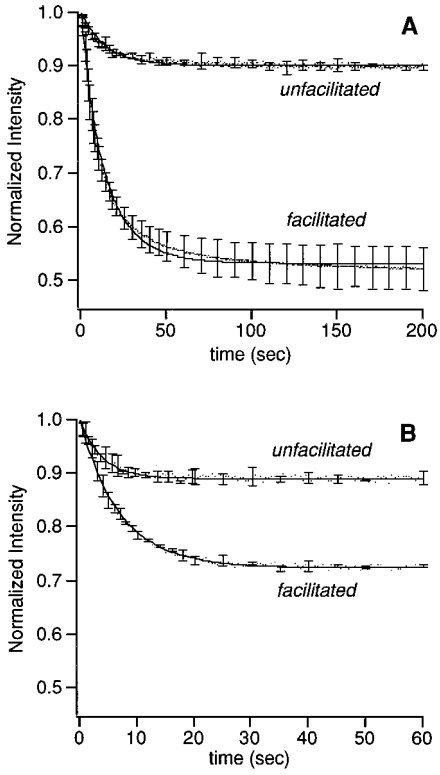

FIGURE 5: Change in 520 nm emission as a function of time after addition of R_{Rh} to S_{Fl} in the absence or presence of facilitator (A) and after addition of R_{Rh} to $P5_{Fl}$ in the absence or presence of facilitator (B). Reaction conditions are given in Materials and Methods. Solid lines represent the nonlinear least-squares fit of the average data (two to four sets) to eq 10 in Materials and Methods. For clarity, error bars representing the standard deviation are given only for points at 5−10 s intervals.

FRET Kinetics. In a typical experiment, 3 mL of a 40 nM solution of the fluorescein-labeled substrate or cleavage product, with or without a 10-fold excess of the facilitator, was prepared in 50 mM Tris-HCl (pH 7.3) with 1 or 20 mM $MgCl_2$ in a fluorescence cuvette equipped with a stir bar. In other experiments, $MgCl_2$ was replaced with 20 mM or 1 M NaCl. The solution was incubated for 15 min at 37 °C with stirring in the spectrophotometer cell block to equilibrate the sample. With the emission monochromator set at 520 nm and the excitation monochromator set at 472 nm, the emission was measured at 0.5 s intervals. After approximately 20 s, 1.4 μL of 83.6 μM tetramethylrhodamine-labeled ribozyme solution was added with an automatic pipettor in a darkened room while continuously collecting emission counts. Emission counts were collected over a period of 10 or 20 min, generating a time profile for the change in fluorescein emission (Figure 5). The time point at which the tetramethylrhodamine-labeled ribozyme was injected was taken as $t = 0$.

The kinetics for this system can be described in terms of the rate of change in the extent of reaction as the system approaches equilibrium (Moore & Pearson, 1981):

$$R_{Rh} + S_{Fl} \underset{k_d}{\overset{k_a}{\rightleftharpoons}} R_{Rh}{\cdot}S_{Fl} \qquad (1)$$

where k_a and k_d are rate constants for association and dissociation, respectively.

The rate expression for this reaction may be given by

$$\frac{dx}{dt} = k_a([R_{Rh}]_0 - x)([S_{Fl}]_0 - x) - k_d([R_{Rh}{\cdot}S_{Fl}]_0 + x) \qquad (2)$$

where $[R_{Rh}]_0$ = the initial ribozyme concentration, $[S_{Fl}]_0$ = the initial substrate concentration, $[R_{Rh}{\cdot}S_{Fl}]_0$ = the initial ribozyme:substrate concentration, and x = the extent of reaction. This equation may be simplified to

$$\frac{dx}{dt} = k_a(r[S_{Fl}]_0 - x)([S_{Fl}]_0 - x) - k_d x \qquad (3)$$

where $r = [R_{Rh}]_0/[S_{Fl}]_0$ and $[R_{Rh}{\cdot}S_{Fl}]_0 = 0$.

At equilibrium, $dx/dt = 0$ and $x = x_e$. Using the equilibrium conditions and solving eq 3 for k_d

$$k_d = \frac{k_a([S_{Fl}]_0 - x_e)(r[S_{Fl}]_0 - x_e)}{x_e} \qquad (4)$$

Substituting eq 4 into eq 3 and integrating gives eq 5.

$$x = \frac{\beta(1 - Z) - (1 + Z)\sqrt{q}}{2Z - 2} \qquad (5)$$

where

$$\beta = \frac{r[S_{Fl}]_0^2 + x_e^2}{x_e} \qquad (6)$$

$$q = \left(\frac{r[S_{Fl}]_0^2 + x_e^2}{x_e}\right)^2 - 4r[S_{Fl}]_0^2 \qquad (7)$$

$$Z = \exp\left(k_a t\sqrt{q} + \ln\frac{b - \sqrt{q}}{b + \sqrt{q}}\right) \qquad (8)$$

The fraction, f, of unbound substrate and the total fluores-

Table 2: Association (k_a)[a] and Dissociation (k_d)[a] Rate Constants for Complexes of R_{Rh} with S_{Fl} and $P5_{Fl}$[b]

complex	[facilitator] (nM)	[Mg^{2+}] (mM)	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_d (nM)
R_{Rh}·S_{Fl}	0	20.0	$(2.10 \pm 0.03) \times 10^5$	0.0537 ± 0.0009	256 ± 6
R_{Rh}·S_{Fl}	400	20.0	$(1.24 \pm 0.01) \times 10^6$	0.0151 ± 0.0001	12.2 ± 0.1
R_{Rh}·S_{Fl}	400	1.0	$(5.97 \pm 1.82) \times 10^4$	0.0064 ± 0.0012	110 ± 40
R_{Rh}·$P5_{Fl}$	0	20.0	$(9.54 \pm 0.18) \times 10^5$	0.189 ± 0.004	198 ± 6
R_{Rh}·$P5_{Fl}$	400	20.0	$(1.41 \pm 0.01) \times 10^6$	0.0599 ± 0.0004	42.5 ± 0.5

[a] k_a refers to the appropriate association rate constants k_1 and k_5 (Figure 1) with substrate and 5′-product, respectively. k_d refers to the corresponding dissociation rate constants k_{-1} and k_{-5}. [b] Rate constants with standard deviations were determined by fitting the average of two to four curves to eq 10.

cence, I_T, at any time are given by eqs 9 and 10, respectively:

$$f = \frac{[S_{Fl}]_0 - x}{[S_{Fl}]_0} \quad (9)$$

$$I_T = fI_S + (1 - f)I_{R\cdot S} \quad (10)$$

where I_S = the fluorescence signal originating from substrate and $I_{R\cdot S}$ = the fluorescence signal originating from substrate bound to ribozyme (Morrison & Stols, 1993).

Experimental data were fitted to eq 10 using the program Igor (Wavemetrics, Lake Oswego, OR). Six parameters were used to fit the data: r, x_e, $[S_{Fl}]_0$, k_a, I_S, and $I_{R\cdot S}$. The values r, $[S_{Fl}]_0$, I_S, and $I_{R\cdot S}$ were held constant. This analysis allowed determination of both k_a and k_d for R_{Rh}·S_{Fl}. The same approach was used to determine rate constants for R_{Rh}·$P5_{Fl}$ (Table 2).

Before the data were fitted to eq 10, it was necessary to determine $I_{R\cdot S}$, the residual fluorescein emission at 100% hybridization of R_{Rh} with S_{Fl} or $P5_{Fl}$. To ensure complete hybridization, the 3′-end of R_{Rh} was lengthened to match the 5′-end of S_{Fl} and $P5_{Fl}$ so as to form a continuous duplex of 19 base pairs with both of these. Two equivalents of this extended ribozyme gave complete hybridization, and no further decrease in the fluorescein emission was observed using a greater excess. Maximum reduction in fluorescein emission in the case of S_{Fl} was 19.5%. With $P5_{Fl}$, reduction in fluorescein emission was 26%. For comparison, reduction in fluorescein emission of 22% was found when S_{Fl} was hybridized to a perfectly complementary oligonucleotide with 26 bases and tetramethylrhodamine at the 5′-end.

Determination of k_2. The apparent value of k_2 (Figure 1) was determined under single-turnover conditions using a 5-fold excess of R_{Rh} over S_{Fl}*. In a typical reaction, a 50 μL solution containing 2 μM S_{Fl}*, a trace amount of ^{32}P-labeled S_{Fl}*, 50 mM Tris-HCl (pH 7.3), and 20 mM $MgCl_2$ was heated at 92 °C for 3 min and then cooled to 37 °C over 20 min. A separate 50 μL solution containing 10 μM R_{Rh}, 50 mM Tris-HCl (pH 7.3), and 20 mM $MgCl_2$ was also heated at 92 °C for 3 min and then cooled to 37 °C over 20 min. For $t = 0$, 5 μL of the S_{Fl}* solution was taken and added to 95% formamide containing 20 mM EDTA and 2 mM Orange G tracking dye. An equal volume of the R_{Rh} solution was then added to the S_{Fl}* solution to initiate the cleavage reaction. After 4, 8, 12, 40, 80, 120, 400, and 800 s, a 5 μL aliquot was taken, added to 5 μL of 95% formamide loading buffer, and placed on ice. After the last time point was collected, each sample was heated at 92 °C for 3 min and then cooled on ice. Samples were analyzed by electrophoresis on 15% polyacrylamide gel containing 8 M urea at pH 8.5. Following autoradiography, substrate and product bands were excised and quantitated on a Beckman LS 6500 scintillation counter (Fullerton, CA). The value of k_2 was determined from the slope of a semilog plot of the fraction of uncleaved substrate versus time (Uhlenbeck, 1987).

Table 3: Michaelis−Menten Kinetic Analysis of Reactions with and without a Facilitator Oligonucleotide[a]

facilitator	k_{cat} (s^{-1})	K_m (nM)	k_{cat}/K_m (s^{-1} M^{-1})
absent	0.040	770	5.08×10^5
present	0.023	98	23.6×10^5

[a] Values were determined from Eadie−Hofstee plots as described in Materials and Methods.

Michaelis−Menten Kinetics. Initial rates of cleavage were determined at five different substrate concentrations ranging from 0.05 to 4 μM in 50 mM Tris-HCl, (pH 7.3) and 20 mM $MgCl_2$ at 37 °C in the presence and absence of the oligonucleotide facilitator in a 2-fold molar excess over the substrate. R_{Rh} concentrations were held constant at 0.025 μM. A typical reaction mixture contained 11 μL of 0.125 μM R_{Rh}, 6 μL of 0.5 M Tris-HCl (pH 7.3), 2 μL of 0.2 mM ^{32}P-labeled S_{Fl}*, an appropriate amount of S_{Fl}* (stock concentrations varied from 10 to 2.5 μM), and enough water to bring the volume to 50 μL. The samples were heated at 92 °C for 3 min and then kept at 37 °C for 20 min. A 5 μL aliquot was taken as the zero time control, and 5 μL of 20 mM $MgCl_2$ was added to the remaining 45 μL to give a solution with a final concentration of 20 mM $MgCl_2$. Aliquots of 5 μL taken at 15, 30, 45, and 60 s were quenched and analyzed as described in the previous section. Plots of product concentration versus time gave straight lines, with slopes equal to the initial rate of reaction. K_m and k_{cat} were determined from an Eadie−Hofstee plot of the data (Table 3).

RESULTS

Effect of Conjugated Dyes on Ribozyme Kinetics and Hybridization. The use of FRET to follow ribozyme kinetics requires fluorescent markers attached to the ribozyme and substrate. From melting curves of complementary oligonucleotides with and without fluorescent labels, we determined that both tetramethylrhodamine and fluorescein contributed to the free energy of hybridization and that these contributions were roughly additive (Table 1).

However, the presence of the markers did not affect the kinetics of the ribozyme under investigation here. Rates of single- and multiple-turnover reactions were the same within experimental error whether the ribozyme and substrate were labeled with fluorophores (data not shown). All subsequent studies reported here were performed using the fluorescently labeled oligonucleotides shown in Figure 3.

FRET Studies. Oligonucleotides labeled with fluorescein show an emission band with a maximum at 520 nm. Addition of 1 equiv of R_{Rh} to S_{Fl} or $P5_{Fl}$ gave 10% reduction in the intensity of this band and the appearance of another

at 580 nm due to tetramethylrhodamine (Figure 4). In the presence of the facilitator, the reduction in fluorescein emission was increased further to 28% in the case of $P5_{Fl}$ and to over 50% in the case of S_{Fl}. No reduction in fluorescein emission of $P3_{Fl}$ by R_{Rh} was seen irrespective of whether the facilitator or 5′-cleavage product was present.

No reduction in fluorescein emission of S_{Fl} resulted from the addition of a noncomplementary, tetramethylrhodamine-labeled oligonucleotide. Therefore, there is no quenching due to collisional effects or absorption of fluorescein emission by tetramethylrhodamine.

In order to quantify complex formation, the extent of reduction in fluorescein emission following complete hybridization was determined. This was done using labeled complementary oligodeoxynucleotides as described in Materials and Methods.

When the magnesium ion concentration was lowered to 1 mM, R_{Rh} and S_{Fl} did not form a complex. Inclusion of the facilitator restored hybridization as indicated by the reduction in fluorescein emission of over 20% (data not shown).

Determination of Rate Constants Using FRET. Time courses for the hybridization of R_{Rh} to S_{Fl} and $P5_{Fl}$ are given in panels A and B of Figure 5. By fitting these to eq 10, rate constants for association and dissociation reactions were determined using magnesium ion concentrations of both 20 and 1 mM (Table 2).

Qualitatively, the effects of the facilitator on binding of R_{Rh} to S_{Fl} and $P5_{Fl}$ were similar. Association rates were increased and dissociation rates decreased, the latter effect being the greater.

The dissociation constant, K_d, is given by

$$K_d = \frac{k_d}{k_a} \tag{11}$$

In 20 mM magnesium, the facilitator decreased the K_d of both $R_{Rh}{\cdot}S_{Fl}$ and $R_{Rh}{\cdot}P5_{Fl}$. However, while $R_{Rh}{\cdot}S_{Fl}$ was stabilized about 20-fold, $R_{Rh}{\cdot}P5_{Fl}$ showed a change of only 5-fold.

In the absence of facilitator, identical kinetics were obtained using S_{Fl}', a truncated form of S_{Fl} lacking the facilitator binding site. Hence, this sequence in the substrate did not engage in secondary structure that could inhibit the rate of complex formation.

Magnesium is important for the formation of the complex $R_{Rh}{\cdot}S_{Fl}$. Lowering the concentration of $MgCl_2$ from 20 to 1 mM eliminated hybridization, and it was not restored by concentrations of NaCl as high as 1 M. However, even in 1 mM $MgCl_2$, the complex formed if the facilitator was present. In these conditions, the stability of the resulting complex was intermediate between those formed in 20 mM Mg^{2+} with and without facilitator (Table 2).

Determination of k_2. The rate constant k_2 for cleavage (Figure 1) was determined under single-turnover conditions using the cleavable substrate S_{Fl}* labeled with ^{32}P. A 5-fold excess of R_{Rh} in 50 mM Tris-HCl (pH 7.3) and 20 mM $MgCl_2$ was added to the substrate in 50 mM Tris-HCl (pH 7.3) and 20 mM $MgCl_2$ to initiate cleavage. (A 5-fold excess of the ribozyme is sufficient to achieve saturation since a 20-fold excess gave the same result.) The reaction was followed by electrophoresis as described in Materials and Methods. The half-life of the substrate was found to be 35 s, giving a value for k_2 of 0.020 s^{-1}. The facilitator had no effect on k_2, as had been found previously (Goodchild, 1992; Nesbitt & Goodchild, 1994).

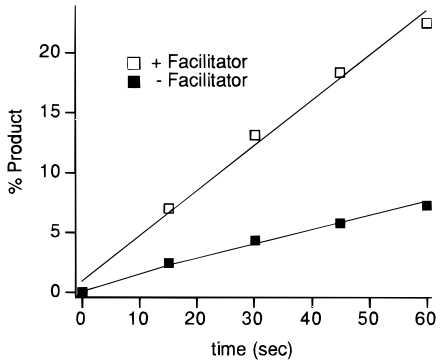

FIGURE 6: Representative time courses of the percent of the product formed in the cleavage of S_{Fl}* by R_{Rh} in the presence (open squares) and absence (closed squares) of facilitator. In general, reactions were carried out in 50 mM Tris-HCl (pH 7.3) and 20 mM $MgCl_2$ at 37 °C in a volume of 50 μL. For the time courses illustrated here, $[S_{Fl}] = 0.050$ μM, $[R_{Rh}] = 0.025$ μM, and $[F] = 0.10$ μM.

Determination of Michaelis−Menten Parameters K_m and k_{cat}. The cleavable form of the substrate S_{Fl}* labeled with ^{32}P was used for Michaelis−Menten analysis of kinetics in the presence and absence of the facilitator. In a typical reaction using excess S_{Fl}* over R_{Rh}, the facilitator increased the initial rate of the reaction by 4-fold (Figure 6). Initial rates of cleavage were determined at substrate concentrations ranging from 0.05 to 4.0 μM with a ribozyme concentration of 0.025 μM. From an Eadie−Hofstee plot, k_{cat} and K_m were obtained (Table 3). The 5-fold increase in k_{cat}/K_m in the presence of facilitator is due mostly to a reduction in K_m; k_{cat} undergoes little change.

DISCUSSION

The purpose of the present work was to investigate the factors that limit the turnover of a hammerhead ribozyme and to reveal how a facilitator oligonucleotide overcomes these. To determine individual rate constants in the hammerhead ribozyme pathway, we developed a method for following the processes in real time using FRET.

Of the 12 rate constants in Figure 1, 10 involve hybridization or dissociation interactions of the various components. Rate constants involving R_{Rh}, S_{Fl}, and $P5_{Fl}$ were determined using FRET, but binding of $P3_{Fl}$ was too weak to be detected. Cleavage was treated as irreversible due to the slow rate of ligation by hammerhead ribozymes (Hertel et al., 1994) and the very rapid dissociation of P3 in the present example. Consequently, k_2 alone was determined under single-turnover conditions.

Michaelis−Menten kinetic studies were also performed for comparison. Values of k_{cat} obtained with and without facilitator were similar to each other and to k_2. The decrease in the Michaelis−Menten constant, K_m, due to the facilitator agrees qualitatively with the decrease in K_d observed by FRET. The Michaelis−Menten analysis thus supports our earlier suggestion that the facilitator improves catalytic efficiency (k_{cat}/K_m) by stabilizing binding to the substrate and so reducing K_m (Goodchild, 1992; Nesbitt & Goodchild, 1994).

From the FRET data, it can be seen that addition of a 1 equiv of R_{Rh} to S_{Fl} or $P5_{Fl}$ gave incomplete hybridization that increased in the presence of the facilitator. This may be explained by a cooperative stacking interaction between

the ends of the facilitator and ribozyme that promotes complex formation. There are several examples of such interactions between neighboring oligonucleotides, and binding was enhanced 1000-fold in some cases (Pitha & Ts'o, 1969; Kutyavin et al., 1988; Maher & Dolnick, 1988; Distefano et al., 1991; Bordier et al., 1992; Azhikina et al., 1993; Kotler et al., 1993; Colocci & Dervan, 1994; François et al., 1994; Walter et al., 1994; Fedorova et al., 1995).

An alternative explanation is that the facilitator may disrupt secondary structure within the substrate that otherwise impedes binding of the ribozyme. This possibility was addressed using S_{Fl}', a truncated form of S_{Fl} lacking the facilitator binding site (Figure 3D). Both forms of the substrate gave the same reduction in fluorescein emission and rate constants for hybridization with R_{Rh}. Thus, binding of R_{Rh} to S_{Fl} was not hampered by secondary structure involving the sequence to which the facilitator binds.

The fluorescein emission of $P3_{Fl}$ was not reduced upon addition of R_{Rh} under the usual conditions. This result was expected as the calculated melting temperature of the duplex that would be formed was less than 0 °C (Freier et al., 1986). Conceivably, the cleavage products might bind cooperatively to R_{Rh}. This proved not to be the case as no reduction in fluorescein emission was observed when R_{Rh}·P5, with or without facilitator, was added to $P3_{Fl}$.

Presumably, both flanking sequences of the ribozyme formed base pairs with the substrate as S_{Fl} hybridized more strongly than either of the products. However, when acting independently, only one formed a stable duplex under the conditions used here. The weak binding of the 3′-cleavage product should result in faster product dissociation and hence greater catalytic turnover of the ribozyme.

Rate enhancement by the facilitator is best understood by comparing values of k_{-1} with and without facilitator (Table 2) with k_2 (0.020 s^{-1}). Without the facilitator, $k_{-1} = 0.0537$ s^{-1} and so the R_{Rh}·S_{Fl} complex dissociates faster than it undergoes cleavage to products ($k_{-1} > k_2$). In the presence of the facilitator, $k_{-1} = 0.0151$ s^{-1} and so is less than k_2. Thus, the complex generates products faster than it dissociates. *This appears to be the key factor in the overall reaction rate enhancement by the facilitator oligonucleotide.*

However, the facilitator also stabilizes the complex R_{Rh}·$P5_{Fl}$ which could result in slower product dissociation and reduced catalytic turnover. Hence, the facilitator affects different parts of the reaction scheme in opposing ways. Previously, we showed that the outcome varied with the length of the flanking sequences so that the reaction of a ribozyme with 10 bases in each flanking sequence was slower in the presence of a facilitator (Nesbitt & Goodchild, 1994). In the present work, the cleavage reaction was slower than dissociation of $P5_{Fl}$ even with the facilitator ($k_{-5} = 0.0599$ s^{-1}) so overall, the facilitator increased catalytic efficiency.

It is possible that the presence of the fluorophores used in FRET may influence the processes under study. We found here that both fluorescein and tetramethylrhodamine contribute to the free energy of oligonucleotide hybridization (Table 1). This should not affect single-turnover reactions which are independent of K_d values. It is more likely that the fluorophores would affect the rates of multiple-turnover reactions. This was not observed in the example studied here because stem I is so unstable. No complex could be detected between $P3_{Fl}$ and R_{Rh}, so for practical purposes, the fluorophores made no change to the dissociation of the 3′-cleavage product. In a ribozyme with a more stable stem I, the fluorophores could well affect the rate of multiple turnover.

The kinetics for hybridization of R_{Rh} with S_{Fl} or $P5_{Fl}$ in Table 2 are expected to be first-order for each component. When a facilitator was used, it was equilibrated with the substrate before initiating the cleavage reaction. The calculated free energy of formation for binding of the facilitator to the substrate is −24 kcal mol^{-1} (Sugimoto et al., 1995). Hence, for the purposes of this study, formation of this complex may be considered irreversible and cleavage kinetics should be independent of the concentration of facilitator provided it is in large enough excess.

For bimolecular cleavage reactions of synthetic hammerhead ribozymes, the optimum concentration of magnesium ion is 20 mM or above (Uhlenbeck, 1987; Pyle, 1993). Cleavage is very poor in concentrations of 1 mM or below such as are found in eukaryotic cells. It has been suggested that magnesium may have both structural and catalytic functions (Pyle, 1993). However, some studies have found no structural role for magnesium in formation of the hammerhead complex (Odai et al., 1990; Heus & Pardi, 1991; Hodgson et al., 1994). For the ribozyme in the present study, the flanking sequences were reduced in length almost to the minimum that would be expected to permit binding to the substrate (Nesbitt & Goodchild, 1994). Since magnesium was required for binding of the ribozyme to the substrate and cannot be replaced by sodium, it most likely had a structural role in the formation of this particular hammerhead complex.

Previously, we found a facilitator was effective in stimulating cleavage in 1 mM magnesium ion and suggested that the facilitator may compensate when concentrations of magnesium are less than optimum for stabilizing the flanking helices (Nesbitt & Goodchild, 1994). The results reported here support this idea. In 1 mM magnesium ion, no complex was formed between the ribozyme and substrate unless the facilitator was present. It increased the stability of the complex in 1 mM magnesium ion above that in 20 mM magnesium without the facilitator. This effect could be useful in improving ribozyme activity in living cells.

We anticipate that the nature of the interactions described here involving facilitators and ribozymes will be general. However, the consequences of these interactions on ribozyme kinetics will vary from case to case according to the stabilities of stems I and III. It is our experience with ribozymes similar to the one described here that the beneficial effects of facilitators are widespread. There are suggestions that, with longer substrates, there may be additional mechanisms for facilitators, possibly by making target sites more accessible to ribozymes (Denman, 1996; Nesbitt & Goodchild, 1994).

In summary, we have shown how a facilitator oligonucleotide improved the catalytic efficiency of a ribozyme by decreasing the rate of dissociation of the ribozyme−substrate complex prior to the chemical step of the reaction. These studies were performed using a new method based on FRET to follow in real time the individual hybridization steps in the reaction pathway. By helping to stabilize duplexes formed by the flanking sequences, the facilitator improved catalytic performance in low concentrations of magnesium such as are found within living cells.

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