Dynamics of a Group I Ribozyme Detected by Spectroscopic Methods

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1 Introduction

Little is known about RNA dynamics, even though it is likely that dynamics are important for both folding and function. The ribozyme, L-21 ScaI, derived from the group I intron of *Tetrahymena thermophila* (Zaug et al. 1988; Kay and Inoue 1987) provides an excellent system for studying dynamics, since its secondary structure is known (Michel and Dujon 1983; Burke et al. 1987; Cech et al. 1994) and a good model is available for its three-dimensional structure (Michel and Westhof 1990). Moreover, spectroscopic probes have been developed that are sensitive to binding of substrate by this ribozyme (Sugimoto et al. 1989b; Bevilacqua et al. 1992; Kierzek et al. 1993). This permits detection of intermediates and measurement of rate constants for various interconversions. The effects of substitutions and of solution conditions on these rate constants give insights into relationships between structure and dynamics and function. The RNA motion most intensively studied thus far in this system is docking of substrate into the catalytic core of the ribozyme (Bevilacqua et al. 1992, 1993, 1994; Li et al. 1995; Li, Profenno and Turner, unpubl. results). This chapter reviews the methods and results of these studies, and discusses some future perspectives.

2 Fluorescent Probes of Dynamics

The most commonly used spectroscopic techniques for studies of RNA dynamics are absorption (LeCuyer and Crothers 1994) and fluorescence (Bevilacqua et al. 1992; Kierzek et al. 1993) These techniques have several advantages: they can detect movement in the absence of chemical reaction; they permit rapid collection of many time points with high precision after initiation of a change, and the time resolution is limited only by the time required for initiation of the change. Thus, in principle, processes as fast as ms and ns can be probed by rapid mixing (Johnson 1992) and temperature-jump (Turner 1986) experiments, respectively.

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Fluorescence is particularly useful for studies of the group I ribozyme, since it can be used with low concentrations of ribozyme where solubility and aggregation are not problems. Since the ribozyme is not naturally fluorescent, the origin of any fluorescence change is localized to the site of the fluorescent probe. Two proven fluorescent probes, pyrene (pyr) and epsilon A (εA), are shown in Fig. 1. The fluorescence of pyrene-labeled substrates, e.g., pyrCCUCU, is enhanced by as much as 20-fold upon binding to ribozyme (Bevilacqua et al. 1992; Kierzek et al. 1993), probably due to diminished quenching by cytosine and uracil (Kierzek et al. 1993). This quenching is apparently due to electron transfer (O'Connor et al. 1994; Netzel et al. 1995; Manoharan et al. 1996). The fluorescence of εA can change by twofold upon binding (Sugimoto et al. 1989b). Fig. 2 provides an example of the wealth of data available from a single shot in a rapid mixing apparatus employing fluorescence detection. In this experiment, pyrCCUCUA was mixed with the group I ribozyme, L-21 ScaI, in the presence of pG. Collection of a total of 500 time points over two separate time windows of 10 and 100 s permitted detection of three separate fluorescence transients. This indicates at least four states of the fluorescent probe in solution, consistent with the proposed mechanism shown in Fig. 2 (Bevilacqua et al. 1994). As discussed below, the first step of the mechanism is base-pairing of substrate with the internal guide sequence of the ribozyme. The second step is docking of the newly formed helix into the catalytic core of the ribozyme, and the third step is reaction to give the products pyrCCUCU and GA. Rates for all three steps can be determined from analysis of this single trace (see Fig. 2). Rate constants for all three steps can be obtained from similar measurements as a function of substrate concentration combined with related chase experiments in which dissociation of complex is followed by rapidly mixing preformed complex with a large excess of an unlabeled oligopyrimidine substrate (Bevilacqua et al. 1994).

A disadvantage of using fluorescent probes is that introduction of the probe may perturb the system under study. Thus it is important to check as

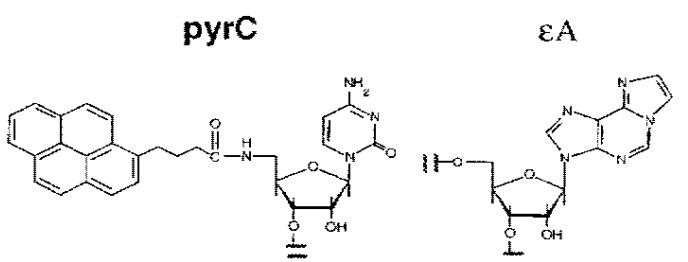

Fig. 1. Structure of the 5′ end of a pyrene-modified oligomer (pyrC), and structure of epsilon adenosine (εA) incorporated into an oligomer

many characteristics as possible against results with less perturbing probes such as ^{32}P. For example, for the pyrene-labeled substrates of the group I ribozyme under solution conditions of 5 mM Mg^{2+}, 135 mM NaCl, 15 °C, the following comparisons suggest that the probe is relatively unperturbing (Bevilacqua et al. 1992, 1993, 1994; Bevilacqua and Turner 1991): For the following reaction at pH 6.5:

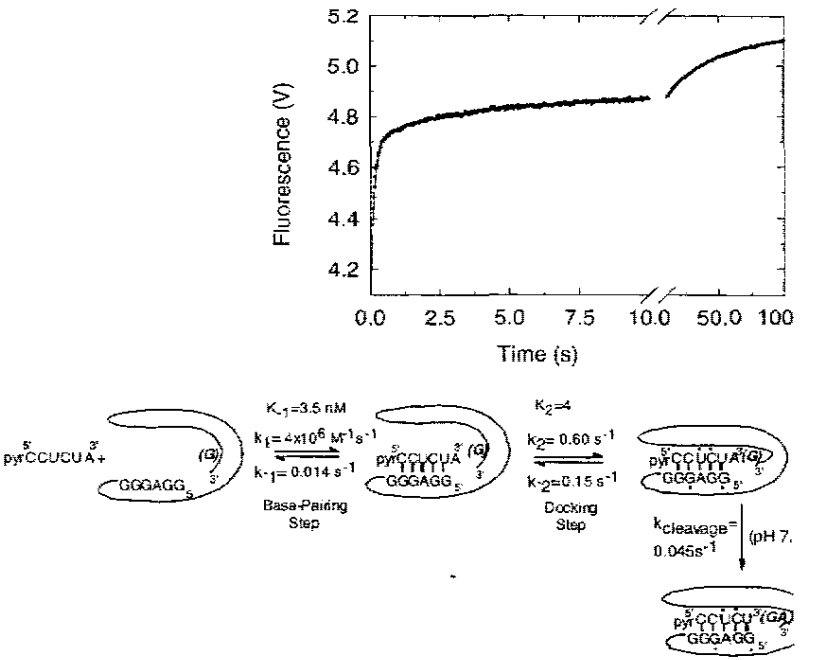

Fig. 2. Dependence of fluorescence (F) on time after mixing equal volumes of pyrCCUCUA/5 mM pG and L-21 Sca I/5 mM pG. Final concentrations after mixing are 2.4 μM pyrCCUCUA, 200 nM L-21 Sca I, and 5 mM prG. Data are the average of two separate mixings. Data are fit to $F = F_\infty + F_1e^{-t/\tau_1} + F_2e^{-t/\tau_2} + F_3e^{-t/\tau_3}$ resulting in rates of $1/\tau_1 = 8.5\,s^{-1}$, $1/\tau_2 = 0.75\,s^{-1}$, $1/\tau_3 = 0.027\,s^{-1}$, and amplitudes of $F_1 = -0.59$, $F_2 = -0.12$, and $F_3 = -0.33$. Buffer is 50 mM HEPES, pH 7.5 with 5 mM $MgCl_2$ and 135 mM NaCl. Also shown is the minimal mechanism for pyrCCUCUA binding to L-21 Sca I in the presence of pG. *Lines* indicate base-pairing. *Bold dots* indicate nucleotides that require 2′OH groups for optimal binding (Sugimoto et al. 1989a; Bevilacqua and Turner 1991; Pyle and Cech 1991; Strobel and Cech 1993)

$$CCUCUA + pG \rightarrow CCUCU + GA, \quad (1)$$

the rates for CCUCUA and pyrCCUCUA are 0.009 and 0.0014 s^{-1}, respectively (Bevilacqua et al. 1994). This sixfold perturbation is modest considering the total catalytic enhancement of about 10^{11} over the uncatalyzed rate (Herschlag and Cech 1990) (see also Herschlag and Cech, this Vol.). For reactions similar to the following:

$$CUCU + UCGA \rightarrow CUCUA + UCG, \quad (2)$$

(k_{cat}/K_m) for UCGA reacting with CUCU and pyrCUCU at pH 7.4 are both 120 $M^{-1}s^{-1}$. For reaction with CCUCU and pyrCCUCU, they are 130 and 220 $M^{-1}s^{-1}$ (Bevilacqua et al. 1992). Thus, pyrene does not have large effects on reactions that mimic both the first and second steps of splicing. In the presence of pdG, the dissociation constant for CUCU measured by equilibrium dialysis is 25 nM, whereas that for pyrCUCU measured by fluorescence detected stopped flow is 230 nM. Pulse-chase experiments indicate that in the absence or presence of pG or pdG there is less than a twofold change in off rate for CUCU and pyrCUCU (Bevilacqua et al. 1993). Tertiary interactions with the longer substrate pyrCCUCU in the absence of pdG enhance binding by 125-fold, identical to the enhancement expected for CCUCU in the absence of pdG (Bevilacqua et al. 1992). Apparently, pyrene is less perturbing with longer substrates, where it is further from the sites of chemistry and tertiary interactions. Finally, qualitative features of the interactions of substrate with ribozyme that have been observed by fluorescence measurements have been confirmed by studies with ^{32}P-labeled substrates (see below).

3 Results from Fluorescence Studies

The initial fluorescence-detected stopped flow studies of group I ribozyme dynamics demonstrated that binding of pyrCUCU, pyrCCUCU, and pyrCUCUCU substrates occurs in two steps as shown in Fig. 3 (Bevilacqua et al. 1992). This two-step binding was most apparent from the observation of two fluorescence transients for binding of pyrCCUCU. A plot of the rates of these fluorescence transients as a function of substrate concentration provides values for k_1, k_{-1}, and $k_2 + k_{-2}$, as shown in Fig. 3. The value of $k_2 + k_{-2}$ can be partitioned into the individual rate constants based on the rate measured in a chase experiment in which preformed ribozyme·substrate complex is mixed with a large excess of unlabeled CCUCU as shown in Fig. 4. When $k_{-1} >> k_{-2}$, the observed dissociation rate, $k_{off} = k_{-2}k_{-1}/(k_{-1} + k_2)$. Thus, with pyrCCUCU, all four rate constants can be determined for the mechanism shown in Fig. 3. A slightly different situation is observed for pyrCUCU. In this case, only a single fluorescence transient is observed, and the apparent k_{on} of 5.8×10^6 $M^{-1}s^{-1}$ is roughly 100-fold slower than that measured for pyrCCUCU. This is because the first intermediate formed with pyrCUCU is relatively unstable so that its concentration never builds up to an observable level. In this case, as

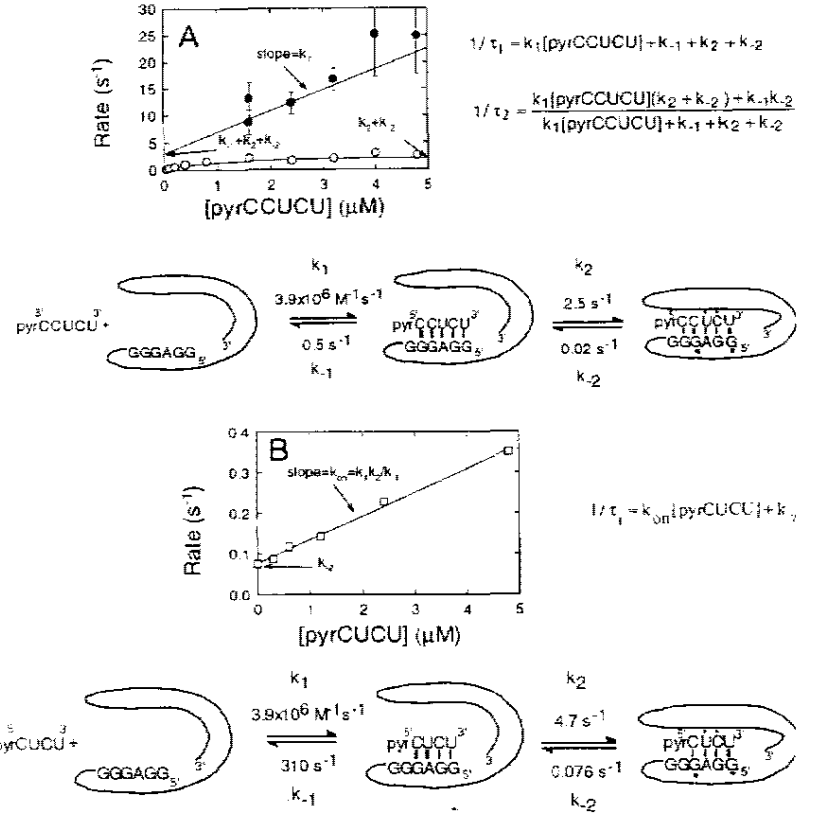

Fig. 3A,B. The dependence of rate on oligomer concentration is shown along with relevant mechanism and rate equations for **A** pyrCCUCU and **B** pyrCUCU. Also shown on rate plots are features that are related to specific rate constants

shown in Fig. 3, the apparent $k_{on} = k_1k_2/k_{-1}$, and the apparent $k_{off} = k_{-2}$. Thus pyrCUCU provides a convenient substrate for studying the effects of various conditions on k_{-2}. Note, however, that if only pyrCUCU had been studied, the minimal mechanism consistent with the data would have been one-step binding with an association rate much slower than expected for helix formation. Thus it is sometimes important to study the kinetics of a series of substrates in order to deduce the correct mechanism.

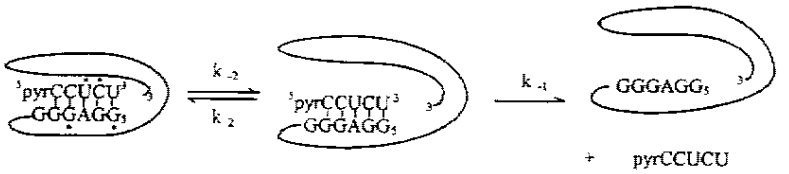

Fig. 4. Scheme for chase experiment described in text

The dissociation constant, $K_d = k_{-1}/k_1$, for the first step in binding pyrCCUCU to ribozyme is 130 nM, essentially the same as the K_d of 120 nM for pyrCCUCU binding to GGAGGA, an oligonucleotide mimic of the internal guide sequence of the ribozyme that base pairs with substrate. This suggests that the first step in binding involves base-pairing of substrate to the ribozyme (Bevilacqua et al. 1992). This had been inferred previously from the absolute magnitude of the association rate measured with ^{32}P-labeled substrates (Herschlag and Cech 1990). Both association and dissociation rates measured by stopped flow for pyrCCUCU binding to ribozyme, however, are about tenfold slower than for binding to the oligonucleotide GGAGGA. This suggests the internal guide sequence is partially occluded in the three-dimensional structure of the ribozyme so that the solid angle for approach and dissociation of substrate is restricted (Bevilacqua et al. 1992).

A second step in binding has been inferred from observations of miscleavage of substrates by a mutant of the L-21 ScaI ribozyme (Herschlag 1992). Presumably, this second step is docking of the initially formed helix into the catalytic core of the ribozyme. Based on the three-dimensional model of Michel and Westhof (1990), this docking is a helix-bundling step. Fluorescence-detected stopped flow provides the first measurements of the rate constants for this RNA folding step. At 15 °C in 5 mM Mg^{2+}, 135 mM NaCl, as shown in Fig. 3, the docking and undocking rate constants, k_2 and k_{-2}, for pyrCCUCU are 2.5 and 0.02 s^{-1}, respectively (Bevilacqua et al. 1992). The rate of docking is roughly 1000-fold slower than rates measured for intramolecular formation of secondary structure. This is consistent with the often used assumption that secondary structure can be predicted without considering tertiary interactions (Turner et al. 1988).

Measurements of the rate constants for docking and undocking make it possible to investigate the factors that affect this RNA folding step. Thus far, the effects of temperature, of extending the oligomer substrate to include a cleavage site, and of occupation of the G binding site of the ribozyme have been investigated.

The effects of temperature on substrate docking have been studied with pyrCUCU (Li et al. 1995). For this substrate, a plot of the rate observed for the

single fluorescence transient as a function of pyrCUCU concentration provides k_2k_1/k_{-1} from the slope and k_{-2} from the intercept (Fig. 3). Since k_1/k_{-1} is the association constant for the base-pairing step, it can be approximated by the association constant for pyrCUCU binding to GGAGAA, an oligonucleotide mimic of the internal guide sequence. The latter association constant as a function of temperature can be obtained from thermal melting experiments. Thus such experiments combined with rates for binding pyrCUCU to ribozyme measured as a function of temperature can provide the temperature dependence of k_2 and k_{-2}. The results of this analysis are summarized in Fig. 5. The activation enthalpy for docking is 22 kcal/mol. This indicates that docking is not a simple diffusion process, since diffusion is associated with activation enthalpies of about 4 kcal/mol. The activation entropy for docking is a favorable 21 eu, suggesting that the transition state is less ordered than the preceding state. This is relatively rare, since transition states are usually more ordered than the preceding state. For example, the activation entropy of undocking is an unfavorable 16 eu. One possible origin for the unusual activation entropy for docking is dehydration of metal ions in the transition state preceding the docked state. For example, Mg^{2+} binding to ATP is associated with a favorable activation entropy of 12 eu (Banyasz and Stuehr 1973). It is quite possible that bundling of helixes would set up specific sites for coordination of Mg^{2+}. A thiophosphate substitution study of ribozyme identified several phosphates important for reaction, including some likely to coordinate Mg^{2+} (Christian and Yarus 1993). The enthalpy and entropy changes for docking of pyrCUCU are 8 kcal/mol and 37 eu, respectively (see Fig. 5). These unfavorable enthalpy and favorable entropy changes are also consistent with dehydration driving docking. For example, Mg^{2+} binding to ATP is associated with a ΔH° and ΔS° of 3 kcal/mol and 28 eu, respectively (Banyasz and Stuehr 1973).

While a fluorescence transient attributable to docking is readily apparent when pyrCCUCU is rapidly mixed with ribozyme at 15 °C and 5 mM Mg^{2+}, no such transient is observed with pyrCCUCUA under the same conditions (Bevilacqua et al. 1994). Rather, a single exponential fluorescence enhancement is observed. Moreover, a plot of the rate of the single fluorescence transient observed for pyrCUCUA vs. pyrCUCUA concentration gives an apparent k_{on} of 3×10^6 M^{-1} s^{-1}, 50-fold faster than for pyrCUCU. Both observations suggest that docking into the catalytic core is unfavorable when A is added to the 3′ end of the substrate. This was initially surprising, since the phosphodiester between U and A is the site of chemistry for the reaction shown in Fig. 2. When preformed ribozyme·pyrCCUCUA complex is mixed with pG, however, two fluorescence transients are observed. These are attributed to docking followed by chemical reaction. Thus pG is able to induce docking. These observations led to a model in which there is an unfavorable tertiary interaction with the terminal pA. Addition of pG provides sufficient additional favorable tertiary interactions to permit docking. The unfavorable tertiary interactions, however, serve to raise the free energy of the ribozyme-

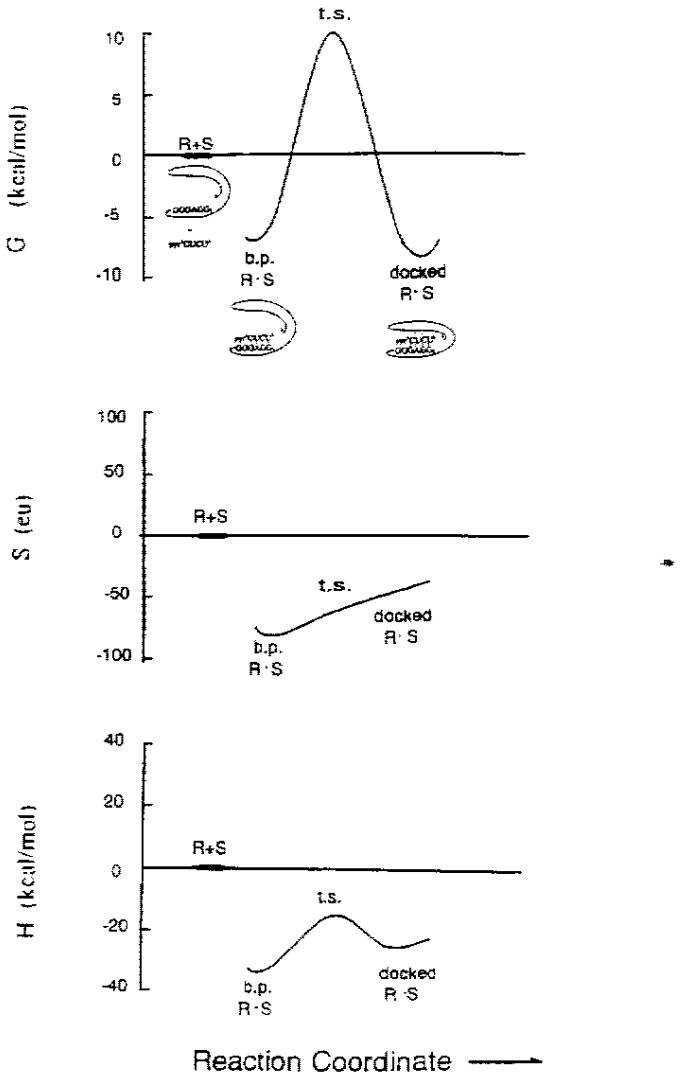

Fig. 5. Free energy at 15 °C, G, entropy, S, and enthalpy, H, versus reaction coordinate for pyrCUCU binding to L-21 Sca I in 50 mM HEPES, pH 7.5, with 5 mM $MgCl_2$ and 135 mM NaCl. The transition state between only base-paired and fully docked complex is represented by *t.s*

substrate complex closer to the free energy of the transition state for chemical reaction. As illustrated in Fig. 6, if these unfavorable interactions are relieved in the transition state, then this provides a catalytic strategy for enhancing reaction rate (Bevilacqua et al. 1994). A similar model has been proposed from studies of substrates labeled with ^{32}P (Narlikar et al. 1995). Such substrate destabilization is known to be important for catalysis by proteins (Jencks 1975; Fersht 1985). It has also been suggested for reaction at the 3′ intron-exon junction of the group I ribozyme based on equilibrium dialysis measurements, showing that UCdGA binds more weakly than UCdG (Moran et al. 1993).

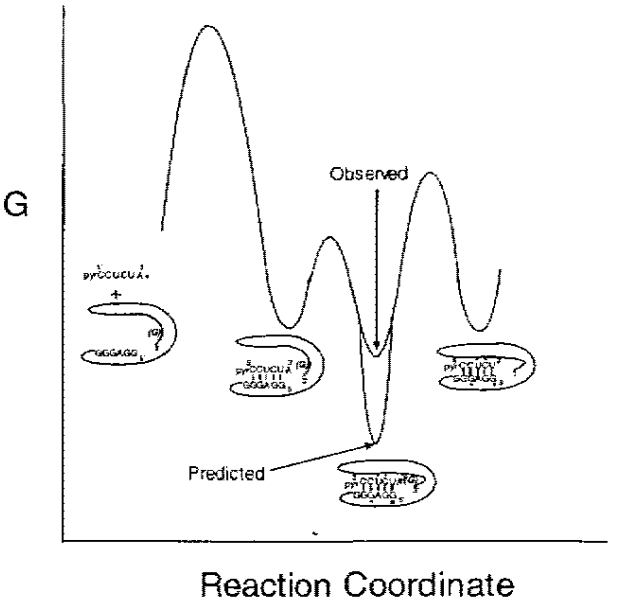

Fig. 6. Free energy at 15 °C, G, versus reaction coordinate for pyrCCUCUA binding to L-21 Sca I in the presence of saturating pG in 50 mM HEPES, pH 7.5, with 5 mM Mg^{2+} and 135 mM NaCl. Shown are the free, base-paired, and docked states for pyrCCUCUA, and the docked state for pyrCCUCU. The predicted free energy for the docked state of pyrCCUCUA is the free energy expected if the tertiary interactions observed with pyrCCUCU docked into the catalytic site of L-21 Sca I are added to the free energy for pyrCCUCUA only base-paired to L-21 Sca I. The measured free energy for pyrCCUCUA docked into the catalytic site is higher (more unfavorable), suggesting substrate destabilization is a catalytic strategy for the ribozyme (Bevilacqua et al. 1994; Narlikar et al. 1995). A similar strategy has been suggested for ribozyme reactions mimicking splicing at the 3′ end of the intron (Moran et al. 1993)

The dramatic effect of the presence of pG on the dynamics of binding pyrCCUCUA is a clear indication of interaction between the two binding sites. The first indication of such interaction came from the observation that pdG enhances the binding of pyrCCUCU by about 1 kcal/mol (Bevilacqua et al. 1992). An interaction between the two binding sites was not initially observed in experiments with ^{32}P-labeled substrates because "coupled binding that gives a small effect of ≤threefold would not have been detected" (Herschlag and Cech 1990). This illustrates two of the advantages of spectroscopic probes: (1) rapid collection of many data points allows high precision in determination of rates, and (2) separation of individual steps on the time axis makes it easier to determine if some intermediate step has been affected by sample preparation. More detailed experiments with ^{32}P-labeled substrates have shown coupled binding between oligopyrimidine substrates and pG (McConnell et al. 1993). Interestingly, fluorescence studies show that the coupled binding is dependent on the 2'OH group of pG (Bevilacqua et al. 1993; Y. Li, L. Profenno, R. Kierzek and D.H. Turner, unpubl. experiments). For example, compared with the absence of cofactor, pyrCUCU binds fourfold more tightly in the presence of 15 mM pdG due to a slower undocking rate, but fourfold more weakly in the presence of 15 mM pG due to a slower apparent on rate (Bevilacqua et al. 1993). Whereas pG induces enhancements of fluorescence that can be attributed to docking of pyrCCUCUA (Bevilacqua et al. 1994) and CCUCUεA (L. Profenno, R. Kierzek, and D.H. Turner, unpubl. experiments), pdG induces quenching of fluorescence, indicating a conformational change to a different state (Y. Li, L. Profenno, R. Kierzek, and D.H. Turner, unpubl. experiments). This indicates that the 2'OH group of G is crucial for correct positioning of substrate in the active site. For a wide variety of conditions, pdG does not support splicing (Bass and Cech 1986) or reactions of type (1) and (2) shown above (Moran et al. 1993). Interestingly, an OH group of tyrosine is important for positioning the reactive phosphodiester bond in the exonuclease site of Klenow fragment (Freemont et al. 1988; Beese and Steitz 1991). It has been suggested that the mechanism for bond cleavage by Klenow fragment may be related to that used by group I introns (Steitz and Steitz 1993). By analogy with the Klenow fragment and the Michel and Westhof (1990) model for the group I intron poised for the second step of splicing, a model structure that is consistent with the importance of the 2'OH group of G in positioning oligopyrimidine substrates prior to the first step of splicing is presented in Fig. 7. In this model, a hydrogen bond between the 2'OH of G and the R_p nonbridging phosphate oxygen at the cleavage site helps hold the reactive phosphodiester bond in place. Note, however, that models with indirect coupling or a combination of direct and indirect coupling are also possible.

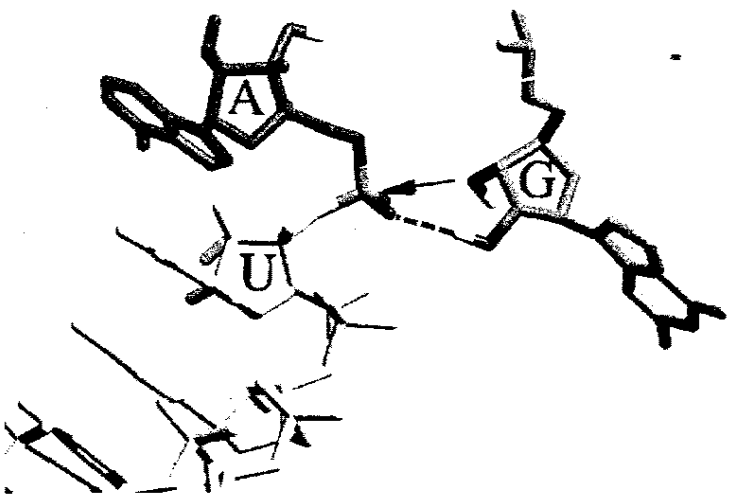

Fig. 7. A speculative model for the interaction of pG with CCUCUA at the catalytic site of the group I ribozyme, L-21 Sca I. *Dashed line* is a possible hydrogen bond between the 2'OH of pG and the R_p nonbridging oxygen between U and A. The *arrow* shows where the 3' oxygen of pG must attack to give the cleavage products CCUCU + GA

4 Future Perspectives

It is likely that the folding and functions of RNA often require large conformational changes. The dynamics of these changes are therefore of considerable interest. Spectroscopic probes can provide much detail concerning dynamics. This chapter has focused on one particular conformational change in one particular RNA. The results relied on changes in the fluorescence of a single fluorophore attached to substrate. It is also possible, however, to use two different fluorophores as an energy donor-acceptor pair (Beardsley and Cantor 1970; Cardullo et al. 1988; Perkins et al. 1993; Tuschl et al. 1994). Fluorescence energy transfer for such a pair is inversely proportional to the sixth power of the distance between donor and acceptor (Forster 1959). Thus, studies employing such pairs should be exquisitely sensitive to dynamics that change the distance between the two fluorophores. Other probes, including absorption (LeCuyer and Crothers 1994), chemical modification (Banerjee and Turner, 1995), and chemical cross-linking (Wang et al. 1993; Wang and Cech 1994) should also be useful. Thus it should be possible to fashion probes for many different conformational changes in many different RNAs. The results should lead to the general principles that govern RNA dynamics.

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