Fluorescence-Detected Stopped Flow with a Pyrene Labeled Substrate Reveals That Guanosine Facilitates Docking of the 5′ Cleavage Site into a High Free Energy Binding Mode in the *Tetrahymena* Ribozyme†

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ABSTRACT: Fluorescence-detected stopped flow kinetics are reported for binding of pyrene (pyr) labeled oligonucleotide substrates, pyrCUCUA and pyrCCUCUA, to the L-21 ScaI ribozyme from *Tetrahymena thermophila*. Both oligomer substrates contain a UA sequence that mimics the cleavage site where pG attacks the self-splicing group I intron from which the ribozyme was derived. Kinetics were measured in the presence and absence of saturating 5′-monophosphate guanosine substrate (pG) at 5 mM Mg^{2+} and 15 °C. In the absence of pG, binding of both oligonucleotide substrates is consistent with a one step mechanism involving only base pairing. Upon addition of pG, pyrCCUCUA is observed to bind in two steps: base pairing to the ribozyme to form the P1 helix and, presumably, subsequent docking of the P1 helix into the catalytic core. A third transient is also observed, which likely includes the chemical step following docking. All rate constants are measured for this mechanism. Surprisingly, the equilibrium constant for docking, K_2, is unfavorable in the absence of pG ($K_2 < 1$) and only modestly favorable in the presence of pG ($K_2 = 4$). These results contrast with those for a 5′ exon mimic, pyrCCUCU, in which docking is strongly favored under the above conditions in the absence of pG; $K_2 = 100$ [Bevilacqua, P. C., Kierzek, R., Johnson, K. A., & Turner, D. H. (1992) *Science 258*, 1355–1358]. These results suggest an unfavorable interaction between the ribozyme and the pA at the site of cleavage. Implications are discussed for the catalytic strategy of the ribozyme and for the self-splicing cascade that occurs in nature.

The discovery of catalysis by RNA (Kruger et al., 1982; Guerrier-Takada et al., 1983; Zaug & Cech, 1986) has led to investigations of the mechanism by which RNA is able to carry out this function (Sugimoto et al., 1988; Herschlag & Cech, 1990; Fedor & Uhlenbeck, 1992; Smith & Pace, 1993; Perreault et al., 1991). One important aspect of catalysis is the recognition and binding of substrates. The L-21 ScaI ribozyme derived from the self-splicing intron of *Tetrahymena thermophila* (Inoue & Kay, 1987; Zaug et al., 1988) acts as an enzyme, catalyzing reactions between exogenously added substrates. Thus it provides a convenient model system for studying these recognition processes. It has been shown that oligonucleotide substrates bind to the L-21 ScaI ribozyme in two steps: base pairing to the complementary internal guide sequence, IGS, to form a helix termed P1, and subsequent docking of P1 into the catalytic core of the ribozyme (Bevilacqua et al., 1992; Herschlag, 1992; Wang et al., 1993). Recently, it has been reported that the oligonucleotide and G binding sites on this ribozyme exhibit cooperative and anticooperative binding depending on the substrates (Bevilacqua et al., 1992, 1993; McConnell et al., 1993). For example, binding of guanosine 5′-monophosphate, pG, weakens binding of a pyrene labeled substrate, pyrCUCU (Bevilacqua et al., 1993), whereas binding of CCCUC(dU)A enhances binding of pG (McConnell et al., 1993). These effects may be important in directing splicing in the natural intron (McConnell et al., 1993; Bevilacqua et al., 1993) because G attacks between U and A in the sequence CUCUCUAAA (Cech et al., 1981).

Rapid reaction kinetics with a pyrene (pyr) labeled substrate (Kierzek et al., 1993) permit dissection of complex binding processes into individual steps, providing insight into the dynamics of RNA catalysis (Bevilacqua et al., 1992). In this paper, we show that there is a fluorescence change associated with cooperative binding between pyrCCUCUA and pG at 5 mM Mg^{2+} and 15 °C. This permits kinetic studies of the step providing cooperativity. The kinetic results suggest cooperativity under these conditions arises because docking of pyrCCUCUA into the catalytic core of the ribozyme changes from unfavorable in the absence of pG to modestly favorable in the presence of pG. These results contrast with those for pyrCCUCU, where docking is strongly favorable in the absence of pG (Bevilacqua et al., 1992), and with those for pyrCUCU where docking is strongly favorable in the absence of pG but less favorable in the presence of pG (Bevilacqua et al., 1993). The results suggest an energetically unfavorable interaction between the pA cleavage site in pyrCCUCUA and the catalytic core of the ribozyme. This unfavorable interaction may contribute to the catalytic strategy of the ribozyme and to the ordering of steps in the splicing cascade.

MATERIALS AND METHODS

Materials. L-21 ScaI was prepared as described previously (Zaug et al., 1988; Bevilacqua & Turner, 1991). It was renatured in 5 mM $MgCl_2$, 135 mM NaCl, and the appropriate buffer for the pH of the experiment (see below) by heating to 50 °C for 10 min and then incubating at 15 °C for 10 min. L-21 ScaI was further incubated at least another 10 min at 15 °C in the presence of pG, if necessary.

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Oligonucleotides modified with pyrene were chemically synthesized as previously described (Kierzek et al., 1993), except that the 2′ hydroxyls of all but the 5′-terminal nucleotide were protected as the *tert*-butyl dimethylsilyl ether (Usman et al., 1987). After machine synthesis, oligomers were deblocked by incubating at 55 °C for 24 h in 2 mL of ammonium hydroxide/ethanol (3:1 v/v). The solution was dried in vacuo, and the residue dissolved in 250 μL of pyridine and dried a total of three times. A deblocking solution of 1 M triethylamine hydrogen fluoride (TEAHF) in pyridine was prepared by placing 6 mL of anhydrous pyridine in a 25-mL round bottom flask, adding 400 μL of 50% aqueous HF, drying the solution in vacuo, redissolving in 6 mL of anhydrous pyridine, redrying, and then adding 8.6 mL of anhydrous pyridine and 1.4 mL of triethylamine. This solution was stored in a plastic bottle. The oligomer was then dissolved in the freshly made 1 M TEAHF in pyridine and incubated at 55 °C for 48 h. The solution was dried in vacuo and the residue dissolved in toluene and dried a total of three times. The residue was then dissolved in 2 mL of water, extracted four times with ether, and dried in vacuo. The sample was dissolved in 600 μL of 0.01 M HCl and adjusted to pH 2.0 by addition of 1 M HCl and incubated at 22 °C for 24 h. This solution was neutralized with ammonium hydroxide, the volume reduced to 250 μL in vacuo, and then desalted on a C-18 Sep-Pack (Waters). The oligomers were purified by TLC (He et al., 1991), labeled with pyrene, and further purified as described previously (Kierzek et al., 1993). Unmodified oligonucleotides were synthesized on solid support with silyl chemistry (Usman et al., 1987). The cofactor pG was obtained from Fluka.

Oligonucleotides labeled with ^{32}P were prepared with T4 RNA ligase (Romaniuk & Uhlenbeck, 1983). First p*Ap was generated by incubating 200 μM Ap with [γ-^{32}P]ATP for 1 h at 37 °C in the presence of 2 units/μL T4 polynucleotide kinase (Boehringer) which lacks 3′-phosphatase activity (Cameron et al., 1978). The buffer for this reaction was 50 mM Tris, 10 mM $MgCl_2$, 5 mM DTE, and 0.1 mM spermidine, pH 8.2. The kinase activity was then inactivated by incubating for 20 min at 65 °C. Oligomer, T4 RNA ligase (New England Biolabs), and ligase buffer were added to the reaction mixture to give final concentrations of 12 μM oligomer, 2 units/μL RNA ligase, 50 mM Tris, 10 mM $MgCl_2$, 3.3 mM DTE, 0.07 mM spermidine, 3.5 mM 2-mercaptoethanol, and 0.35 mM ATP. This mixture was incubated for 2 h at 15 °C to allow reaction and then for 15 min at 65 °C to inactivate the ligase. To remove the terminal phosphate, calf intestinal phosphatase (Boehringer) was added to a final concentration of 1 unit/μL and the mixture incubated for 30 min at 37 °C. Products were purified on a 20% polyacrylamide gel run in 0.025 M Tris, 0.02 M boric acid, 0.00025 M sodium EDTA buffer at pH 8.5. Bands were cut out and eluted into water.

Kinetics. All kinetics experiments were performed at 15 °C in 5.0 mM $MgCl_2$ and 135 mM NaCl. Buffers were 50 mM MES (9 mM Na^+), pH 5.5, 50 mM PIPES (50 mM Na^+), pH 6.5, and 50 mM HEPES (25 mM Na^+), pH 7.5. When pG was included, the final concentration was 5 mM. Data were fit with nonlinear least-squares algorithms.

A KinTek stopped-flow apparatus with 1.5-ms mixing time was used for rapid mixing experiments, as described previously (Johnson, 1986; Bevilacqua et al., 1992), except a 395-nm bandpass filter with a 50-nm full-width at half-maximum (FWHM) and a 75-W xenon lamp were used. Identical results were obtained with 380-nm (10-nm FWHM) and 400-nm (10-nm FWHM) filters, but the signal-to-noise ratio was about 3-fold higher with the 395-nm filter. Concentrations of pyrCUCUA and pyrCCUCUA were in excess of L-21 ScaI to maintain pseudo-first-order conditions. Unless stated otherwise, concentrations reported in the text are concentrations immediately after mixing the contents of the two syringes. The final concentration of L-21 ScaI was 20, 40, or 200 nM; higher values were used when possible to improve the signal-to-noise ratio. Five hundred time points were collected for each trace.

The change in fluorescence, F, with time was fit to the sum of a constant, F_∞, and one, two, or three exponentials, as required, according to the following equation:

$$F = F_\infty + F_1 \exp(-t/\tau_1) + F_2 \exp(-t/\tau_2) + F_3 \exp(-t/\tau_3)$$

where F_∞ is F at equilibrium, and F_i and $1/\tau_i$ are, respectively, the amplitude and rate for the ith exponential. Residuals for the fits are shown above the figures of fluorescence data, and χ^2 is given in the figure legends.

Kinetics for ^{32}P-labeled substrates were measured by preincubating about 1 nM substrate with 2 μM L-21 ScaI for 2 min followed by addition of an equal volume of 10 mM pG. Typically, 10 time points spanning six half-lives were taken by removing 3 μL and quenching into 5 μL of 90% formamide/10% aqueous 0.8 M Tris-borate buffer at pH 8.5/0.05% bromophenol blue/0.05% xylene cyanol, and freezing in a dry ice/ethanol bath. If the stop solution was added to one reactant before addition of the other, no reaction was observed. Products and reactants were separated by electrophoresis on a 20% polyacrylamide/8 M urea gel. Bands were quantitated on a Molecular Dynamics Phosphorimager. Reported rates are averages of four determinations with standard deviations.

RESULTS

Binding of pyrCUCUA in the Absence of pG. Figure 1A shows a trace of fluorescence intensity vs time after mixing L-21 ScaI with pyrCUCUA. The trace is single exponential, and the rate of fluorescence increase is about 100-fold faster than previously measured for pyrCUCU at identical concentrations (Bevilacqua et al., 1992). As illustrated in Figure 2B, the slow rate for pyrCUCU has been attributed to a two-step mechanism involving base pairing followed by docking of P1 into the catalytic core (Bevilacqua et al., 1992). Because the base pairing is not saturated and is in rapid equilibrium relative to docking, this leads to an apparent association rate constant $k_{on} = k_1(k_2/k_{-1}) = 5.8 \times 10^4$ M^{-1} s^{-1} for pyrCUCU (Bevilacqua et al., 1992). In contrast, the association rate constant for pyrCUCUA is 3.0×10^6 M^{-1} s^{-1} (Table 1 and Figure 2A), as determined from a linear least-squares fit of rate, τ^{-1}, vs [pyrCUCUA] (Figure 1B) according to the following equation describing a one-step bimolecular mechanism (Figure 2A) with pyrCUCUA in excess (Johnson, 1992):

$$\tau^{-1} = k_1[\text{pyrCUCUA}]_0 + k_{-1} \quad (1)$$

Although the association rate constant for pyrCUCUA is 50-fold faster than for pyrCUCU, it is similar to the k_{on} ($=k_1$) of 3.9×10^6 M^{-1} s^{-1} measured for pyrCCUCU binding to L-21 ScaI (Bevilacqua et al., 1992). In the latter case, k_{on} is the rate constant for the base pairing step.

The intercept of the plot in Figure 1B gives a dissociation rate, $k_{off} = k_{-1}$, of 16 s^{-1} for pyrCUCUA (eq 1), allowing calculation of the equilibrium dissociation constant, $K_d = k_{off}/k_{on} = k_{-1}/k_1 = 5.3$ μM. Comparison with the K_d of 1.3 μM measured for the overall binding of pyrCUCU to L-21 ScaI (Bevilacqua et al., 1992) indicates pyrCUCUA binds to L-21

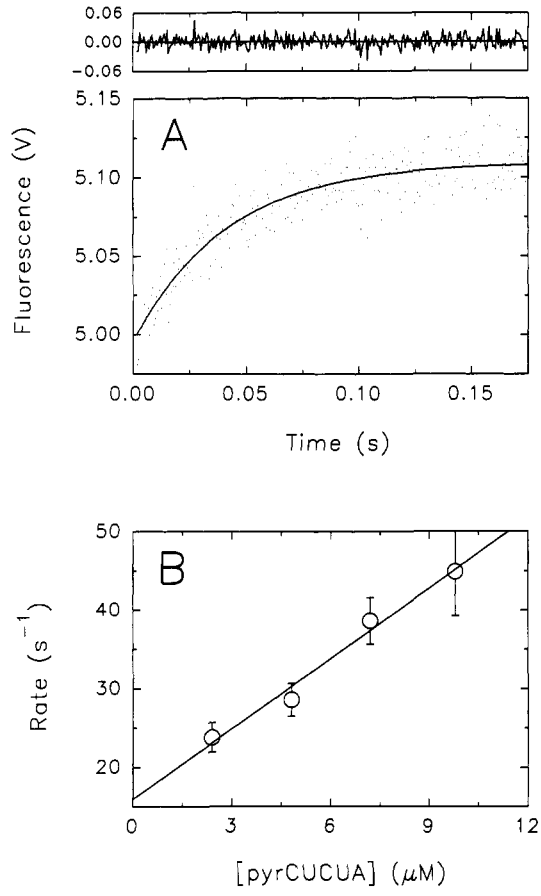


FIGURE 1: (A) Representative trace and fit for dependence of fluorescence intensity, F, on time after mixing equal volumes of pyrCUCUA and L-21 Sca I. Final concentrations after mixing are 2.4 μM pyrCUCUA and 200 nM L-21 Sca I. Data represent the average of three separate mixings. Data are fit to $F = F_\infty + F_1 e^{-t/\tau_1}$ resulting in $1/\tau_1 = 24\ s^{-1}$ with $\chi^2 = 0.04$. Buffer is 50 mM HEPES, pH 7.5. (B) Dependence of rate on pyrCUCUA concentration for binding to 200 nM L-21 Sca I. Data points come from fits of fluorescence versus time to a single exponential, truncated at six half-lives. The line through the data points is by linear regression. Error bars represent standard deviation in rate from fits. Buffer is 50 mM HEPES, pH 7.5.

ScaI more weakly than pyrCUCU. This is surprising since a 3′ unpaired A at the end of a simple duplex is expected to favor helix formation in the first step of binding by a factor of 15 at 15 °C (Freier et al., 1986). The K_d expected for pyrCUCUA forming a simple duplex with the sequence GGAGGA is estimated as 2 μM from extrapolating kinetic results for pyrCUCU binding to GGAGGA at 5 °C (Bevilacqua et al., 1992) and adding the predicted stabilizing effect of the unpaired 3′ A (Freier et al., 1986). [GGAGGA is an analogue of the internal guide sequence, GGAGGG, that binds the oligonucleotide substrate on L-21 ScaI (see Figure 2).] Thus the k_{on} and K_d determined by kinetics are consistent with a model in which pyrCUCUA binds in only one step to L-21 ScaI, forming a simple duplex without enhanced binding from tertiary interactions. This contrasts with pyrCUCU which forms a duplex with L-21 ScaI that is stabilized by a further factor of 60 by tertiary interactions [Figure 2B and Bevilacqua et al. 1992)].

While the mechanism in Figure 2A is the simplest interpretation of the data for pyrCUCUA in the absence of pG, an alternative interpretation in terms of a two-step mechanism like that of Figure 2B is possible. For the general two-step case, $k_{on} = k_1k_2/(k_2 + k_{-1})$. Since k_{on} is similar to the value expected for k_1, this interpretation requires $k_2 > k_{-1}$. In this case, $k_{off} = k_{-1}/K_2$. Thus, if $K_2 = 10$, then $k_{-1} = 10 \times 16 = 160\ s^{-1}$, similar to that expected for pyrCUCU (see Figure 2B). This would imply that the 3′ A of pyrCUCUA is unstacked in the initial complex. Moreover, k_2 would have to be $>160\ s^{-1}$, much larger than the value of $2.5\ s^{-1}$ measured for pyrCCUCU (Bevilacqua et al., 1992). We therefore consider this mechanism unlikely.

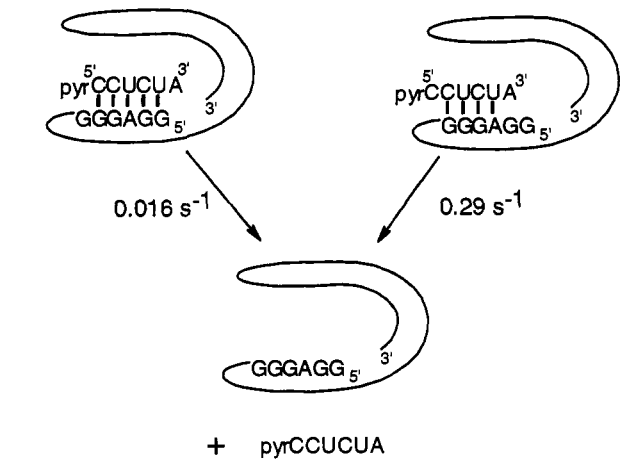
Binding of pyrCCUCUA in the Absence of pG. A characteristic fluorescence trace for pyrCCUCUA binding to L-21 ScaI and a plot of rate as a function of [pyrCCUCUA] are shown in Figure 3. The slope of this plot gives a k_{on} ($=k_1$) of $4.4 \times 10^6\ M^{-1}\ s^{-1}$ (eq 1 and Table 1), consistent with the rates for the base pairing step for pyrCCUCU binding to L-21 ScaI (Bevilacqua et al., 1992) and with k_{on} for pyrCUCUA (see Figures 2A and 4A,C).

The intercept of the plot in Figure 3B is too small to provide an accurate value for k_{off}. If pyrCCUCUA only forms base pairs, then K_d is predicted to be 120/15 = 8 nM, where 120 nM is the measured K_d for pyrCCUCU binding to GGAGGA at 15 °C (Bevilacqua et al., 1992) and the factor of 15 arises from the 15-fold tighter binding expected from the 3′ A (Freier et al., 1986). This leads to a predicted $k_{off} = k_{-1} = k_{on}K_d$ of $0.03\ s^{-1}$, consistent with the small intercept observed.

To measure k_{off}, a chase experiment was performed (Figure 5). In this experiment, the pyrCCUCUA·L-21 ScaI complex was mixed with a large excess of unlabeled CCUCU. The pH of both solutions was 5.5 to suppress potential hydrolysis of pyrCCUCUA. Rates for base pairing and docking at pH 5.5 are expected to be equivalent to the rates at pH 7.5 since Herschlag et al. (1993) report that K_d for $CCCUCUA_5$ is independent of pH from 5 to 7. Moreover, McConnell et al. (1993) report the binding of G in the presence and absence of CCCUC(dU)A is unchanged between pH 5.5 and 7, suggesting the structure of the catalytic core is unaffected in this pH interval. The observed decrease in fluorescence during the chase experiment was double exponential with 80% of the amplitude having a rate of $0.016\ s^{-1}$, close to the predicted rate of $0.03\ s^{-1}$ for dissociation of a simple helix (see above). The remaining 20% of the amplitude had a faster rate of 0.29 s^{-1}. The two off-rates suggest at least two binding modes for pyrCCUCUA. For example, the data are consistent with Scheme 1, in which the faster dissociation rate arises from pyrCCUCUA pairing out of register with the IGS.

The out of register pairing might be able to dock and thus be stabilized by tertiary interactions, whereas the matched pairing is unable to dock. Alternatively, the two complexes might have identical P1 helixes positioned in different locations.

Scheme 1



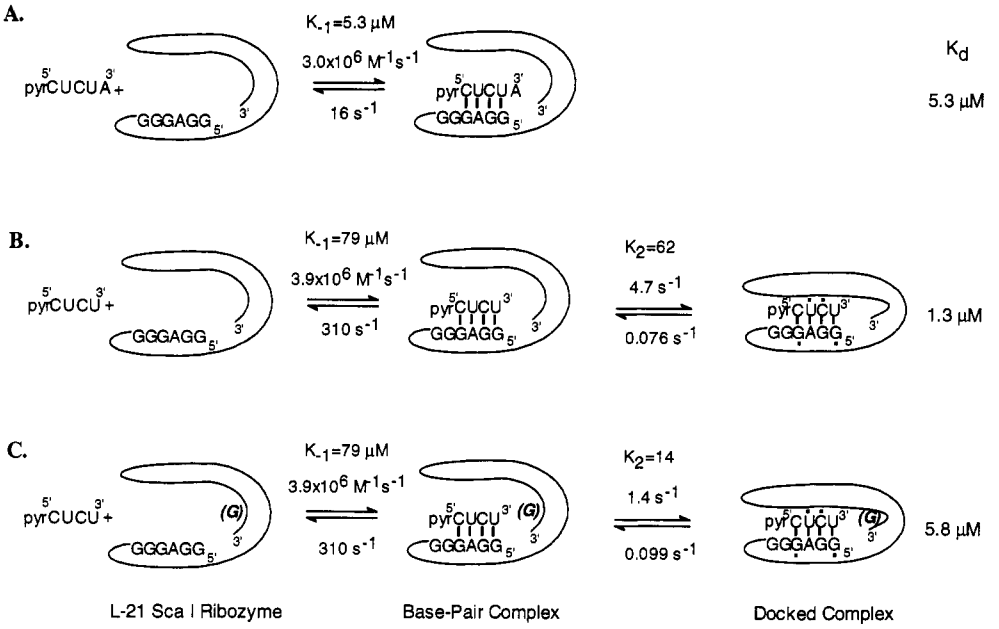


FIGURE 2: Minimal mechanisms for pyrCUCUA and pyrCUCU binding to L-21 Sca I in the presence or absence of pG, as indicated. Lines indicate base pairing (Michel & Dujon, 1983; Waring et al., 1983; Been & Cech, 1986). Bold dots indicate nucleosides that require 2′ OH groups for optimal binding (Pyle & Cech, 1991; Bevilacqua & Turner, 1991; Strobel & Cech, 1993).

Table 1: Kinetic Parameters for Binding to L-21 Sca I, Unless Indicated, Errors Are Roughly 10%

substrate	cofactor	k_1 (μM^{-1} s^{-1})	k_{-1} (s^{-1})	K_{-1} (μM)	k_2 (s^{-1})	k_{-2} (s^{-1})	K_{-2}	K_d (μM)
pyrCUCU[a]	none	3.9[b]	310[c]	80[c]	4.7 ± 2	0.076	0.016	1.3
pyrCUCUA	none	3.0 ± 0.3	16 ± 2	5.3 ± 0.8			≫1	5.3 ± 0.8
pyrCCUCU[a]	none	3.9 ± 0.5	0.5 ± 0.2	0.13 ± 0.06	2.5 ± 0.6	0.02 ± 0.01	0.008 ± 0.005	0.0011 ± 0.0008
pyrCCUCUA	none	4.4	0.016	0.0037			≫1	0.0037
pyrCUCU[d]	15 mM pG	3.9[b]	310[c]	80[c]	1.4	0.099	0.07	5.8
pyrCCUCUA	5 mM pG	4[e]	0.014	0.004	0.60	0.15	0.26	0.001

[a] Bevilacqua et al. (1992). [b] Not measured directly. Assumed to be the same as for pyrCCUCU. [c] Calculated with k_1 assumed in b, and with K_{-1} (80 μM) for this step extrapolated from K_{-1} for pyrCCUCU and nearest-neighbor parameters for helix propagation (Freier et al., 1986; He et al., 1991). [d] Bevilacqua et al. (1993). [e] Value is calculated from the fast transient in Figure 7 and is the same with and without pG.

Recent cross-linking results at 42 °C have indicated more than one location for the unpaired IGS, although there appeared to be only one major location for the undocked P1 helix (Wang et al., 1993).

The ratio of the measured association rate and predominant dissociation rate, 4.4×10^6 M^{-1} s^{-1} and 0.016 s^{-1}, respectively, gives a K_d of 3.7 nM (Table 1), close to the value of 8 nM expected for simple helix formation (see above). Thus kinetic results for both pyrCUCUA and pyrCCUCUA binding to L-21 ScaI in the absence of pG are consistent with formation of a simple duplex without enhanced binding from tertiary interactions.

The Effect of pG on pyrCCUCUA Binding and Reactivity. pG is known to enhance binding of CCCUC(dU)A to L-21 ScaI by a factor of 7 at 10 mM Mg^{2+} (McConnell et al., 1993). The results described above suggest that pyrCCUCUA docking into the catalytic core at 5 mM Mg^{2+} is unfavorable in the absence of pG. Thus we designed several experiments to determine the effect of pG on pyrCCUCUA binding to L-21 ScaI. The effect of pG on the L-21 ScaI·pyrCCUCUA complex was determined by mixing 10 mM pG with an equal volume of 400 nM L-21 ScaI and 1600 nM pyrCCUCUA. The high concentration of pyrCCUCUA assures that all the L-21 ScaI has pyrCCUCUA bound prior to mixing. As shown in Figure 6, two exponentials are observed with roughly equal amplitudes and rates of 0.75 and 0.045 s^{-1}. The rates and relative amplitudes of the two exponentials were unchanged when the pyrCCUCUA concentration was increased 3-fold. When the pH was lowered from 7.5 to 6.5, the rate of the faster exponential was essentially unchanged at 0.7 s^{-1}, whereas the slower exponential was fit moderately well with a rate of 0.0048 s^{-1} (Supplementary Material; see paragraph at end of paper regarding Supplementary Material). Herschlag et al. (1993) have shown that the rate of the cleavage step decreases linearly with $[H^+]$ in this pH range for the related substrates $(dC)_3dUdC\underline{rU}(dA)_5$, $(dC)_3\underline{rU}dCdU(dA)_5$, and $(dC)_3dUdCdU(dA)_5$. This suggests the slower exponential in Figure 6 reports the cleavage step, either directly or upon release of GA.

The rate of the cleavage step was independently measured by mixing 10 mM pG with an equal volume of 2 μM L-21 ScaI containing a trace concentration of pyrCCUCUp*A, where p* denotes a radioactive phosphate. The reaction course as a function of time was followed by separating reactants and products on a 20% acrylamide/8 M urea gel (see Materials and Methods). The rates measured at pH 7.5 and 6.5 are 0.034 ± 0.006 and 0.0014 ± 0.0002 s^{-1}, respectively. The rate at pH 7.5 is within experimental error of the rate for the slowest fluorescence change at pH 7.5, and the rate at pH 6.5 is roughly 3-fold slower than the rate for the slowest fluorescence change at pH 6.5. This is consistent with the assignment of these fluorescence transients to the cleavage step. [Note that rates of cleavage have been seen to vary between independent experiments by factors of 2 for unknown reasons (Herschlag & Cech, 1990; Herschlag et al., 1993)]. The slowest fluorescence change at pH 6.5, however, is fit better by a double exponential having roughly equal amplitude components with rates of 0.008 and 0.0014 s^{-1}, with the latter

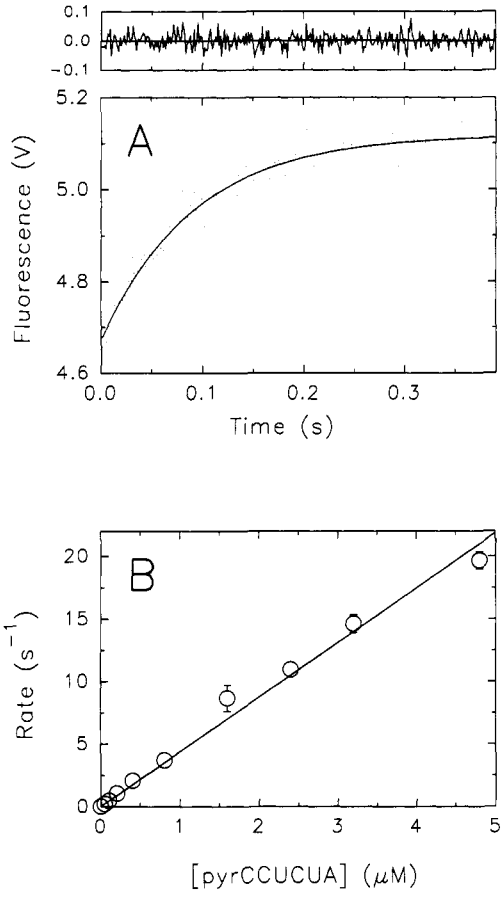


FIGURE 3: (A) Representative trace and fit for dependence of fluorescence intensity, F, on time after mixing equal volumes of pyrCCUCUA and L-21 Sca I. Final concentrations after mixing are 2.4 μM pyrCCUCUA and 200 nM L-21 Sca I. Data are fit to $F = F_\infty + F_1 e^{-t/\tau_1}$ resulting in $1/\tau_1 = 10.6\ s^{-1}$ with $\chi^2 = 0.2$. Buffer is 50 mM HEPES, pH 7.5. (B) Dependence of rate on pyrCCUCUA concentration for binding to 20 (with 50–1600 nM pyrCCUCUA) and 200 nM L-21 ScaI (with 2400–4800 nM pyrCCUCUA). (Concentrations are final after mixing.) Data points come from fits of fluorescence versus time to a single exponential, truncated at six half-lives. The line through the data points is by linear regression and forced through a y-intercept of 0.016 s^{-1}, determined by a pulse–chase experiment in the absence of pG (see text). Error bars represent standard deviation in rate from fits. Buffer is 50 mM HEPES, pH 7.5.

rate that determined for cleavage with the gel method (see Supplementary Material). Thus the fluorescence may detect another step with rate 0.008 s^{-1} at pH 6.5. Either way, the data are consistent with the slowest fluorescence change corresponding to chemical cleavage at both pH 7.5 and 6.5. The rate of cleavage was also measured for CCUCUA (not labeled with pyrene) at pH 6.5 and was 0.009 ± 0.002 s^{-1}, about 6-fold faster than for pyrCCUCUA. At pH 7.5, the rate for CCUCUA cleavage was >0.05 s^{-1}. Evidently, pyrene modestly decreases the cleavage rate.

The above data are fit well by a model in which addition of pG causes at least one conformational change in the L-21 ScaI·pyrCCUCUA complex that is independent of pH between pH 6.5 and 7.5, and the new conformation catalyzes the transesterification reaction (see Figure 4B). Presumably, the conformational change is docking of the P1 helix formed by pyrCCUCUA into the catalytic core. For this mechanism, the rate of the first exponential is $k_2 + k_{-2}$, the sum of docking and undocking rates (Bernasconi, 1976; Johnson, 1992). Both the fluorescence change and the reactivity suggest $K_2 > 1$, so that the observed rate of docking, k_2, can be estimated as ≈0.7 s^{-1}. This value is similar to the rate of 1.4 s^{-1} estimated for pyrCUCU docking in the presence of pG, based on the measured docking rate in the absence of pG (Bevilacqua et al., 1992) and the effect of pG on k_1k_2/k_{-1} (Bevilacqua et al., 1993).

As a control, a volume of 10 mM pG was mixed with an equal volume of 400 nM L-21 ScaI and 1600 nM pyrCCUCU. No fluorescence change was observed. It has been shown that pyrCCUCU docks into tertiary interactions in the absence of pG (Bevilacqua et al., 1992), so the results of this control are consistent with expectations from previous work.

The results above suggest that pyrCCUCUA primarily binds to L-21 ScaI by base pairing alone in the absence of pG, and that subsequent binding of pG induces a conformational change that leads to reaction. Thus, in a single mixing experiment, it should be possible to monitor all three steps involving pyrCCUCUA: base pairing, docking, and chemistry. This was tested by mixing equal volumes of a solution of 400 nM L-21 ScaI and 5 mM pG with a solution of 4.8 μM pyrCCUCUA and 5 mM pG. As shown in Figure 7, the kinetic trace consists of three exponentials with rates of 8.5, 0.75, and 0.027 s^{-1}. The fastest rate is that expected for base pairing (see Figure 3B), assuming pG has no effect on k_1. This suggests the rate for base pairing of pyrCCUCUA is the same to free L-21 ScaI and the L-21 ScaI·pG complex. This is in agreement with the results of McConnell et al. (1993) for CCCUC(dU)A binding at 10 mM Mg^{2+} and pH 5.5. The middle rate is essentially identical to that measured for the conformational change induced by addition of pG to the L-21 ScaI·pyrCCUCUA complex. The slowest rate is similar to that ascribed above to the chemical reaction. Thus this mixing experiment is consistent with the proposed mechanism shown in Figure 4B.

A Chase Experiment at pH 5.5 Provides Rates for Undocking and Docking of pyrCCUCUA. For the mechanism shown in Figure 4B, the above experiments provide values for k_1, k_{-1}, $k_2 + k_{-2}$, and k_3. To determine k_{-2} and k_2 individually, a chase experiment was performed on the pG·L-21 ScaI·pyrCCUCUA complex. Since this complex is reactive, the chase experiment was conducted at pH 5.5 where the rate for the chemical step is expected to be 0.00014 s^{-1}, based on a rate of 0.0014 s^{-1} at pH 6.5 and the observation that this rate is log-linear with pH over this pH range (Herschlag et al., 1993). This corresponds to a half-life of about 80 min, about 20-fold slower than dissociation (see below).

In the chase experiment, a volume of 400 nM L-21 ScaI, 1600 nM pyrCCUCUA, and 5 mM pG was mixed with an equal volume of 20 μM CCUCU and 5 mM pG. A double-exponential decay of fluorescence was observed with rates of 0.014 and 0.0027 s^{-1} (Figure 8). The faster rate is similar to the rate of 0.016 s^{-1} measured for breaking of base pairs in the absence of pG (see above). Thus, this data and that from the previous section indicate the rates of making and breaking base pairs in the presence and absence of pG are similar. This suggests the slower rate, which accounts for 70% of the total amplitude, arises from dissociation from the docked complex so that the overall scheme for the chase experiment is as shown in Scheme 2. For this scheme, the measured slower exponential gives $k_{off} = k_{-1}k_{-2}/(k_{-1} + k_2 + k_{-2})$. Solving for k_{-2} with k_{-1} = 0.014 s^{-1} and $k_2 + k_{-2}$ = 0.75 s^{-1} gives k_{-2} = 0.15 s^{-1} and k_2 = 0.60 s^{-1}. The ratio $k_2/k_{-2} = 4$ provides an equilibrium constant for docking in the presence of pG. This equilibrium constant is consistent with the observation of two exponentials in the chase experiment since both docked and undocked states have significant populations.

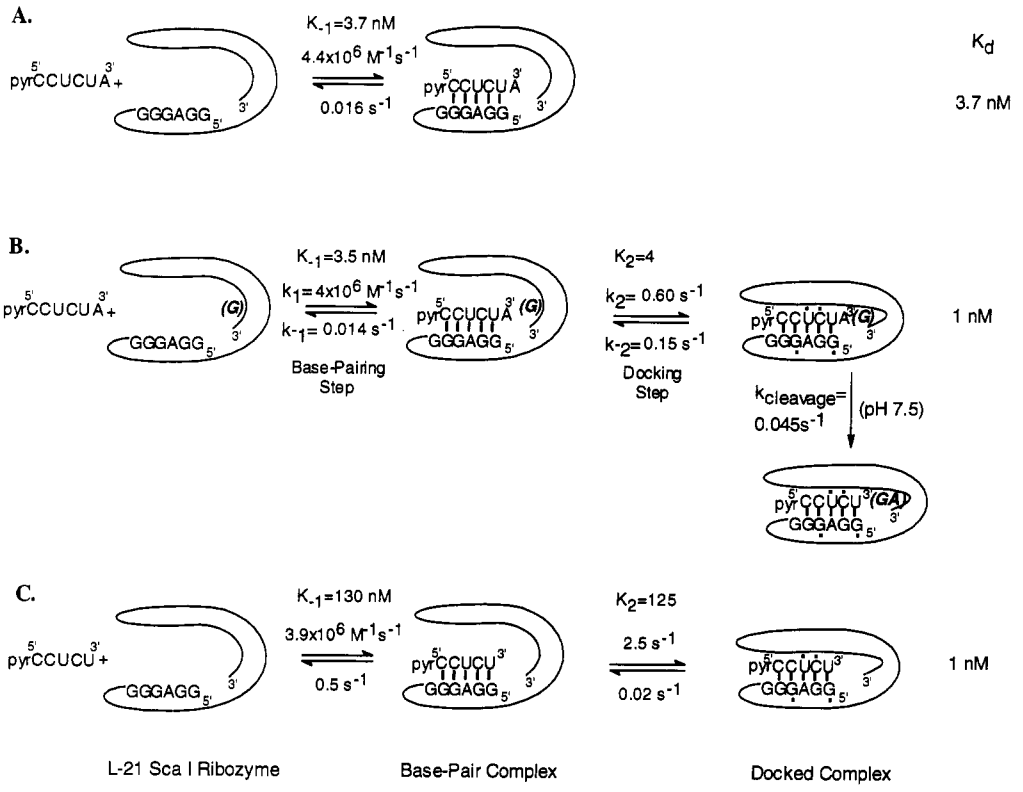
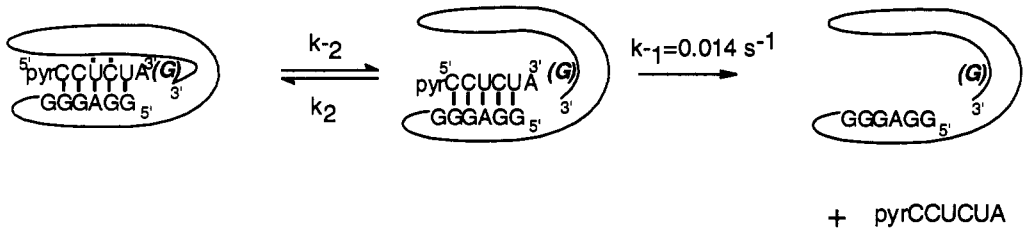


FIGURE 4: Minimal mechanisms for pyrCCUCUA and pyrCCUCU binding to L-21 Sca I in the presence or absence of pG, as indicated. Lines indicate base pairing (Michel & Dujon, 1983; Waring et al., 1983; Been & Cech, 1986). Bold dots indicate nucleosides that require 2′ OH groups for optimal binding (Pyle & Cech, 1991; Bevilacqua & Turner, 1991; Strobel & Cech, 1993).

Scheme 2



DISCUSSION

The simplest interpretation of the experiments described above is summarized in Figures 2 and 4. The most striking conclusion is that when pG is absent, the docking step is favorable for pyrCUCU and pyrCCUCU, but unfavorable for pyrCUCUA and pyrCCUCUA. For example, the equilibrium constants for docking (K_2) of pyrCCUCU and pyrCCUCUA in the absence of pG are 100 ($\Delta G°_{15}$ = –2.6 kcal/mol) and <1 ($\Delta G°_{15}$ > 0 kcal/mol), respectively. This suggests that unfavorable interactions with the pA destabilize docking by 3 kcal/mol or more. The unfavorable effect is similar to the more than 30-fold destabilization of binding ($\Delta\Delta G°_{15}$ > 2 kcal/mol) to L-21 ScaI when UCdG and GUCdG are lengthened to UCdGA, UCdGU, GUCdGA, and GUCdGU (Moran et al., 1993). The latter oligomers are mimics for the 3′ splice and cyclization sites in the *Tetrahymena* intron (Cech & Bass, 1986). Thus addition of either the 5′ or 3′ splice site to its appropriate recognition sequence appears to result in an unfavorable tertiary interaction with the ribozyme. As discussed below, this destabilization of binding in the ground state may increase the rate of transesterification and help order the steps in the splicing cascade.

The unfavorable effect of the 3′ pA on docking of pyrCCUCUA contrasts with the favorable stacking interactions observed for the 3′ unpaired A's at the ends of the oligomer duplex $(GCCGGUAp)_2$ (Freier et al., 1986) and in the base paired complex formed with L-21 ScaI (compare K_1's with and without pA for substrates in Figures 2 and 4). In both cases, each unpaired 3′ A enhances binding by a factor of about 15–30 ($\Delta G°_{15}$ = –1.5 to –2.0 kcal/mol). Thus the unfavorable effect on docking (≥3 kcal/mol) may be even larger than the free energy required to unstack the 3′-terminal A (≈2 kcal/mol).

These conclusions from experiments at 15 °C in 5 mM Mg^{2+} contrast with those for the same ribozyme at 42 and 50 °C in 10 mM Mg^{2+} (Herschlag & Cech, 1990; Pyle et al., 1990). When compared with duplex formation between the oligomers CCCUCU and GGAGGGAAA (S. Moran and D. H. Turner, unpublished results, extrapolated to CCCUCUA), reported binding of $GGCCCUCUA_5$ to the ribozyme at both 42 (Pyle et al., 1990) and 50 °C (Herschlag et al., 1990) is tighter than expected by factors of about 10 and 200, respectively. It is likely the factor at 42 °C is even larger since the incubation time for the experiments at 42 °C was not long enough to achieve equilibrium (Bevilacqua & Turner, 1991; Pyle et al., 1992). This suggests the equilibrium constant

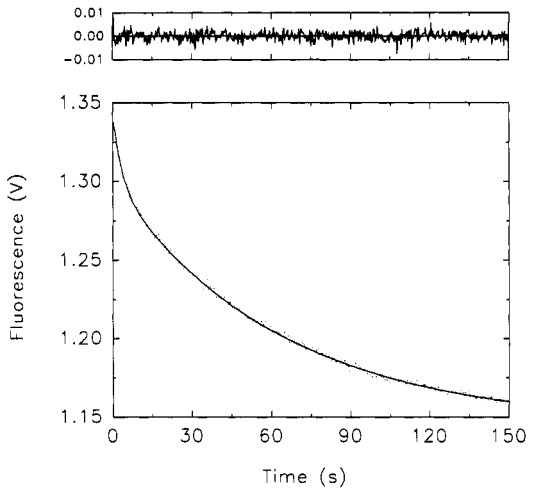


FIGURE 5: Dependence of fluorescence intensity, F, on time after mixing equal volumes of pyrCCUCUA/L-21 Sca I and excess CCUCU. Final concentrations after mixing are 0.8 μM pyrCCUCUA, 200 nM L-21 Sca I, and 10 μM CCUCU. Data are fit to $F = F_\infty + F_1e^{-t/\tau_1} + F_2e^{-t/\tau_2}$ resulting in $1/\tau_1 = 0.29$ s^{-1}, $1/\tau_2 = 0.016$ s^{-1}, $F_1 = 0.039$, and $F_2 = 0.15$, with $\chi^2 = 0.002$. The fitted curve is also shown. Buffer is 50 mM MES, pH 5.5, to slow the rate of chemistry (see text).

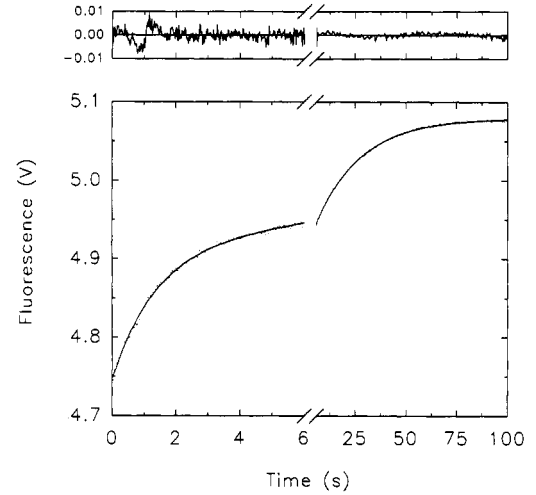


FIGURE 6: Dependence of fluorescence intensity, F, on time after mixing equal volumes of pyrCCUCUA/L-21 Sca I and pG. Final concentrations after mixing are 0.8 μM pyrCCUCUA, 200 nM L-21 Sca I, and 5 mM pG. Data represent the average of three separate mixings. Data are fit to $F = F_\infty + F_1e^{-t/\tau_1} + F_2e^{-t/\tau_2}$ resulting in $1/\tau_1 = 0.75$ s^{-1}, $1/\tau_2 = 0.045$ s^{-1}, $F_1 = -0.16$, and $F_2 = -0.17$, with $\chi^2 = 0.002$. The fitted curve is also shown. Buffer is 50 mM HEPES, pH 7.5

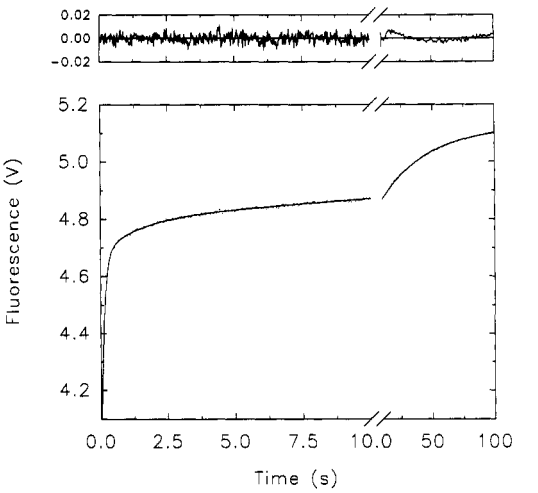


FIGURE 7: Dependence of fluorescence intensity, F, on time after mixing equal volumes of pyrCCUCUA/5 mM pG and L-21 Sca I/5 mM pG. Final concentrations after mixing are 2.4 μM pyrCCUCUA, 200 nM L-21 Sca I, and 5 mM pG. Data represent the average of two separate mixings. Data are fit to $F = F_\infty + F_1e^{-t/\tau_1} + F_2e^{-t/\tau_2} + F_3e^{-t/\tau_3}$ resulting in $1/\tau_1 = 8.5$ s^{-1}, $1/\tau_2 = 0.75$ s^{-1}, $1/\tau_3 = 0.027$ s^{-1}, $F_1 = -0.59$, $F_2 = -0.12$, and $F_3 = -0.33$, with $\chi^2 = 0.006$. The fitted curve is also shown. Buffer is 50 mM HEPES, pH 7.5.

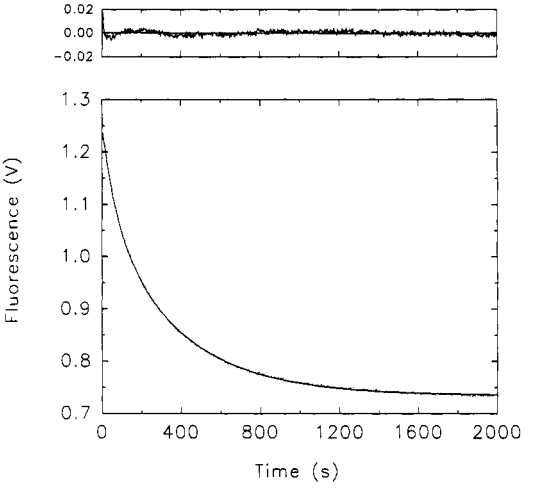


FIGURE 8: Dependence of fluorescence intensity, F, on time after mixing equal volumes of pyrCCUCUA/L-21 Sca I/5 mM pG and excess CCUCU/5 mM pG. Final concentrations after mixing are 0.8 μM pyrCCUCUA, 200 nM L-21 Sca I, 10 μM CCUCU, and 5 mM pG. Data are fit to $F = F_\infty + F_1e^{-t/\tau_1} + F_2e^{-t/\tau_2}$ resulting in $1/\tau_1 = 0.014$ s^{-1}, $1/\tau_2 = 0.0027$ s^{-1}, $F_1 = 0.15$, and $F_2 = 0.36$, with $\chi^2 = 0.002$. The fitted curve is also shown. Buffer is 50 mM MES, pH 5.5, to slow the rate of chemistry (see text).

for docking at the higher temperatures and 10 mM Mg^{2+} is >10. The comparison suggests either the $\Delta H°$ for docking is positive so that docking is more favorable at higher temperature or docking is favored by higher [Mg^{2+}], or both. Experiments on the temperature dependence for binding of pyrCUCU to L-21 ScaI indicate docking is associated with a positive $\Delta H°$ (Y. Li, P. C. Bevilacqua, and D. H. Turner, unpublished results). It is also likely that Mg^{2+} plays a role (Bevilacqua, 1993; Wang & Cech, 1994).

A more complex interpretation of the data at 15 °C and 5 mM Mg^{2+} is possible. For example, a model in which the 3′-terminal pA of the oligomer destabilizes binding in the initial complex and $k_2 \gg k_{-1}$ is also consistent with the data. In this model, the single fluorescence transient observed for binding of oligomer in the absence of pG is due to both the base pairing and docking steps. The fastest fluorescence transient observed upon addition of pG to the preformed ribozyme·oligomer complex then reports a conformational change that results in cooperative binding of docked oligomer substrate and pG. This model requires that the $\Delta G°_{15}$ for docking be similar to the $\Delta G°_{15}$ of destabilization due to the 3′ pA, that the rate for docking pyrCCUCUA be much greater than the rate for docking pyrCCUCU, and that the rate for the conformational change induced by pG be similar to the rate for docking pyrCCUCU. We therefore consider this model unlikely. Both models, however, lead to the conclusion that the terminal 3′ pA of the oligomer destabilizes binding in the docked complex.

Differential Docking May Be Part of the Catalytic Strategy of the Ribozyme. At 15 °C, the equilibrium constant for docking of pyrCCUCUA apparently switches from unfavorable to favorable upon binding of pG (Figure 4). Even in the presence of pG, however, K_2 is only 4 ($\Delta G°_{15} = -0.8$ kcal/mol). This contrasts with values of 100 for pyrCCUCU in

the absence of pG (Bevilacqua et al., 1992) and about 15 for pyrCUCU in the presence of pG (Bevilacqua et al., 1993). Evidently, even in the presence of pG, pyrCCUCUA docks in a high free energy binding mode relative to pyrCCUCU. If some part of the additional favorable free energy associated with docking of pyrCCUCU is gained in the transition state for the chemical step of cleavage of pyrCCUCUA, then the difference in ground state binding between pyrCCUCUA and pyrCCUCU would serve to bring the ground state for pyrCCUCUA closer to the transition state. This substrate destabilization would provide a catalytic strategy for increasing the rate of transesterification (Jencks, 1975; Fersht, 1985; Menger, 1992). Moran et al. (1993) have suggested a similar strategy for the 3′ splice site, and a three-dimensional model of the second step of splicing shows a sharp turn in the backbone at the 3′ splice site (Michel & Westhof, 1990). Strain from such a sharp turn might lead to a high free energy binding mode. The results presented here suggest this same strategy may be used for both the first and second steps of splicing.

The free energy required to introduce a turn at the splice site is probably modest. In particular, $\Delta G°_{15}$ for the UA/UG nearest neighbor interaction at the splice site is only −1.3 kcal/mol, less than any combination of Watson–Crick nearest neighbors except AA/UU (−1.3 kcal/mol) and AU/AU (−1.2 kcal/mol) (He et al., 1991; Freier et al., 1986b). The free energy change for the UA/UG nearest neighbors includes contributions from stacking, hydrogen bonding, and other terms (Freier et al., 1986; Turner et al., 1987). Studies of stacking geometries involving GU mismatches indicate the overlap of the GU is much larger with the base pair to the 5′ side of the U than to the 3′ side of the U (Mizuno & Sundaralingam, 1978; van Knippenberg et al., 1990; White et al., 1992). Thus, if required, it may take relatively little free energy to disrupt stacking between the U and A surrounding the site of transesterification. This suggests that a large part of the ground state binding energy lost upon addition of pA to the 3′ end of the substrate could go into straining or stressing the phosphodiester bond at the splice site. This strain or stress could then be relieved in the transition state.

Comparison with Structural Models. The weak docking of pyrCCUCUA is surprising in light of phylogenetic models for group I introns. In the natural precursor, the nucleotides surrounding the 5′ splice site are thought to be paired with the internal guide sequence in a continuous helix (P1) (Michel & Dujon, 1983; Waring et al., 1983):

5′-CUCUCUAAA-3′
GGGAGGUUU

In addition, a three-dimensional model of the catalytic core of the ribozyme before the *second step* of splicing (Michel & Westhof, 1990) suggests there should be sufficient space in L-21 ScaI to accommodate the stacked 3′ unpaired A of pyrCCUCUA. Thus there is no obvious reason to expect unfavorable steric interactions with the pA at the splice site. Interestingly, however, a model of the exonuclease active site of *Escherichia coli* DNA polymerase I has a leucine that disrupts stacking between the two nucleotides surrounding the cleavage site (Beese & Steitz, 1991). Clearly a ribozyme has numerous unpaired bases that could also be used as a wedge to disrupt stacking at a cleavage site. Alternatively, the ribozyme may be set up to accept pA in an AU base pair which may have a different geometry than a 3′ unpaired pA.

Influence of Docking on the Splicing Cascade. It has been suggested that cooperative and anticooperative interactions between substrates in the splicing reaction may help order the kinetic steps in splicing (Bevilacqua et al., 1993; McConnell et al., 1993). For example, McConnell et al. (1993) showed that binding of CCCUC(dU)A leads to tighter binding of pG and that binding of pG leads to a slower off rate for CCCUC-(dU)A. The results presented here suggest that at 5 mM Mg^{2+} and 15 °C, the weaker binding of pyrCCUCUA in the absence of pG is due to an unfavorable interaction with the cleavage site that forces the equilibrium constant for docking into the catalytic core to be <1. Thus reaction at the cleavage site, e.g., by hydrolysis, is disfavored in the absence of pG. Binding of pG, however, increases the equilibrium constant for docking to 4, thus favoring the first reactive step of splicing. Once pG attacks at the 5′ exon–intron junction to give $CUCUCU_{OH}$, however, it is beneficial both to not bind another pG so that the 3′ splice site, GU, can bind into the G-binding site, and to hold $CUCUCU_{OH}$ in the catalytic site until it reacts with the 3′ intron–exon junction. This is accomplished by increasing the equilibrium constant for docking to roughly 100 in the absence of pG (Bevilacqua et al., 1992), while at the same time employing anticooperative interactions that reduce pG binding by roughly a factor of 5 to the docked complex (Bevilacqua et al., 1993; McConnell et al., 1993). Thus the available results suggest that as splicing proceeds, the equilibrium constant for docking increases from <1 to 4 upon binding of pG, to 100 upon release of the G-intron product. Thus the early steps of splicing are accompanied by a progressive increase in the equilibrium constant for docking. Evidently, the self-splicing intron acts like a minature assembly line using docking equilibria to put parts together in the correct order. Both favorable and unfavorable tertiary interactions are important in this process. It would not be surprising to see the progression reversed for the late steps of splicing, in order to release product.

The Cleavage Step. The slowest transient observed for pyrCCUCUA in the presence of pG appears to include the cleavage step. Evidently, pyrene fluorescence is also sensitive to this step. This change in fluorescence intensity is consistent with the interpretation that cleavage at the splice site leads to changes in the structure of the ribozyme, thus changing the local environment of the pyrene. The sensitivity of pyrene makes it possible to study the effects of various conditions and structural changes on the rates of at least three reaction steps at once (see Figure 7). This should provide a powerful tool for structure–function studies of this and other ribozymes.

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SUPPLEMENTARY MATERIAL AVAILABLE

Two figures of the rapid mixing data at pH 6.5 with the slowest fluorescence transient fit to one and two exponentials, respectively (3 pages). Ordering information is given on any current masthead page.

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