Effects of Mg^{2+} and the 2′ OH of Guanosine on Steps Required for Substrate Binding and Reactivity with the *Tetrahymena* Ribozyme Reveal Several Local Folding Transitions†

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ABSTRACT: Transient kinetic studies with fluorescence detection were used to determine the effects of Mg^{2+} concentration and the 2′ OH group of guanosine monophosphate, prG, substrate on various steps in the transesterification reaction of prG with 5′ pyrene-labeled oligonucleotides as catalyzed by the L-21 *Sca*I ribozyme. The effect of increasing Mg^{2+} from 5 to 10 mM on the rate constants of association and dissociation of 5′ pyrene-labeled CUCUA at 15 °C was measured. The rate constant of association increases about 3-fold to $(8.7 \pm 0.7) \times 10^6$ M^{-1} s^{-1} at 10 mM Mg^{2+}. The rate constant for dissociation is 25 ± 4 s^{-1} at 10 mM Mg^{2+}, within experimental error of the rate constant of 17 ± 5 s^{-1} measured at 5 mM Mg^{2+}. This Mg^{2+} dependence is attributed to nonspecific binding of Mg^{2+} to the duplex helix. In the absence of prG, no docking of substrate is observed. The effect of Mg^{2+} concentration on rates for docking of 5′ pyrene-labeled substrate, pyrCCUCUA, were measured at $[Mg^{2+}] \geq 2$ mM and at temperatures ≤ 20 °C, where optical melting curves indicate global folding is complete. Thus the rates monitor local folding steps important for catalytic function. Three and possibly four local cooperative transitions were induced by Mg^{2+}. The fastest fluorescence transient, which is associated with substrate docking, changes from a quenching to an enhancement between 2 and 4 mM Mg^{2+}, and its observed rate constant at pH 6.5 and 7.5 is about 1 s^{-1}, independent of $[Mg^{2+}]$ when $4 \leq [Mg^{2+}] \leq 15$ mM. The slowest fluorescence transient, which is apparently associated with transesterification, has an observed rate constant that continues to increase when $[Mg^{2+}] \geq 4$ mM. In the presence of Ca^{2+}, such that $[Ca^{2+}] + [Mg^{2+}] = 15$ mM, the observed rate constants of both transients are constant when 4 mM ≤ $[Mg^{2+}]$ ≤ 7 mM but double between 7 and 11 mM Mg^{2+}. At pH 6.5 when 4 mM ≤ $[Mg^{2+}] \leq 7$ mM in the absence of Ca^{2+}, there is also evidence for a third transient with a Mg^{2+}-dependent observed rate constant that is intermediate between the observed rate constants of docking and transesterification. Thus these experiments reveal several separable, local folding transitions that are dependent on Mg^{2+} in a very cooperative manner and are important for function. When pdG is substituted for prG, no transesterification is observed, and fluorescence quenching is observed for 1−15 mM Mg^{2+}. The switch from fluorescence enhancements with prG to quenching with pdG suggests the 2′ OH of prG is important for proper positioning of substrate in the catalytic site.

Understanding the interactions that determine RNA structure and dynamics is important for predicting RNA folding (Turner et al., 1988; Michel & Westhof, 1990; Draper, 1996; Lehnert et al., 1996) and for rational targeting of RNA by therapeutics (Bevilacqua & Turner, 1991; Fourmy et al., 1996; Lima et al., 1992). Crystal structures of tRNA, the hammerhead ribozyme, and the P4−P6 domain of the *Tetrahymena* ribozyme suggest that Mg^{2+} ions and 2′ OH groups are both important for tertiary interactions (Robertus et al., 1974; Ladner et al., 1975; Holbrook et al., 1977; Jack et al., 1977; Sussman et al., 1978; Hingerty et al., 1978; Quigley et al., 1978; Moras et al., 1980; Westhof & Sundaralingam, 1986; Pley et al., 1994; Scott et al., 1995; Cate et al., 1996; Cate & Doudna, 1996). Solution studies have shown the importance of Mg^{2+} for folding of tRNA (Danchin, 1972; Lynch et al., 1974; Römer & Hach, 1975; Stein & Crothers, 1976; Crothers, 1979; Teeter et al., 1980; Bujalowski et al., 1986), the *Tetrahymena* ribozyme (Inoue & Cech, 1985; Latham & Cech, 1989; Grosshans & Cech, 1989; Jaeger et al., 1990; Downs & Cech, 1990; Celander & Cech, 1991; Wang & Cech, 1994; Zarrinker & Williamson, 1994), domains from ribosomal RNA (Laing et al., 1994; Lu & Draper, 1994), and the hammerhead ribozyme (Dahm & Uhlenbeck, 1991; Bassi et al., 1995; Menger et al., 1996). Several RNAs are also known to require Mg^{2+} for catalytic activity (Guerrier-Takada et al., 1986; Grosshans & Cech, 1988; Dahm & Uhlenbeck, 1991; Piccirilli et al., 1993; Smith & Pace, 1993; Beebe et al., 1993; Pan et al., 1993; Pyle, 1993; Yarus, 1993; Pan, 1995) Studies of *Tetrahymena* ribozymes have provided insight into free energy increments available from tertiary interactions involving 2′ OH groups (Sugimoto et al., 1989; Pyle & Cech, 1991; Bevilacqua & Turner, 1991; Strobel & Cech, 1993; Herschlag et al., 1993a). While the importance of Mg^{2+} and 2′ OH groups is clear, much remains to be discovered about the range of motifs involving them and how they contribute to function.

The *Tetrahymena* ribozyme, L-21 *Sca*I (Inoue & Kay, 1987; Zaug et al., 1988), provides an excellent system for studying RNA interactions since it is relatively small, its catalytic activity depends on proper folding, and small

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substrates can be chemically synthesized and added *in trans*. These substrates must then be assembled into the catalytic site for reaction to occur. Thus steps along the reaction pathway can be considered as individual RNA folding steps. For example, oligoribonucleotide substrates, such as CCCUCUA, must first base-pair with an internal guide sequence to form a helix designated P1 (Waring, 1983; Michel & Westhof, 1990; Been & Cech, 1986) and then dock into the catalytic site (Herschlag, 1992; Bevilacqua et al., 1992). The docking can be followed by changes in fluorescence of pyrene-labeled substrates such as pyrCCUCUA (Bevilacqua et al., 1992, 1993, 1994; Li et al., 1995; Turner et al., 1996). It has been shown that pyrCUCUA and pyrCCUCUA will base-pair to form P1 but not dock at 5 mM Mg^{2+}, 15 °C, in the absence of prG (Bevilacqua et al., 1994). This is consistent with a phase diagram developed for CCCUCUA (Narlikar & Herschlag, 1996). Upon addition of guanosine monophosphate, prG, however, pyrCCUCUA, denoted as S, will dock into the catalytic site of the L-21 *Sca*I ribozyme, abbreviated as R, and react to give prGA and pyrCCUCU, denoted as P (Bevilacqua et al., 1994):

$$S{\cdot}R + prG \rightleftharpoons S{\cdot}R{\cdot}prG \rightleftharpoons [S{\cdot}R{\cdot}prG]' \rightleftharpoons P{\cdot}R{\cdot}prGA \quad (1)$$

Here S·R·prG is the base-paired ternary complex and [S·R·prG]′ is the ternary complex with docked substrate. Thus the second step involves intramolecular formation of tertiary interactions, while the third step could be limited by either RNA folding steps or phosphoryltransfer. Transient kinetic methods with fluorescence detection permit studies of these individual steps (Turner et al., 1996). In this paper, we report fluorescence-detected stopped-flow studies of this pathway as a function of Mg^{2+} concentration with prG and pdG substrates. The results suggest optimal reactivity depends on several local folding transitions in the ribozyme that depend cooperatively on Mg^{2+} concentration, that a tertiary interaction with the 2′ OH of prG is required for productive docking of substrate, and that the reaction mechanism may be more complicated than that shown above.

MATERIALS AND METHODS

Materials. L-21 *Sca*I was prepared as described previously (Zaug et al., 1988; Bevilacqua & Turner, 1991). It was renatured by heating to 50 °C for 10 min in the same buffer used for the experiment and then incubated for at least 10 min at the temperature of the experiment.

Oligonucleotides modified with pyrene were chemically synthesized (Kierzek et al., 1993; Bevilacqua et al., 1994), and pyrCCUCUA labeled with ^{32}P was prepared as described by Bevilacqua et al. (1994). The cofactors, prG and pdG, were purchased from Fluka and Sigma, respectively, and used without further purification.

Kinetics. A KinTek stopped-flow instrument was used for rapid mixing experiments as described previously (Johnson, 1986; Bevilacqua et al., 1992, 1994). Final concentrations after mixing were typically 150 nM L-21 *Sca*I, 300 nM pyrCCUCUA, and 5 mM prG or pdG. Buffers were 50 mM PIPES (50 mM Na^+), pH 6.5, or 50 mM HEPES (25 mM Na^+), pH 7.5. Total Na^+ concentration was maintained at 160 mM by adjusting NaCl concentration. Divalent metal ion concentration was varied. In a mixing experiment, both solutions had identical buffer and cation concentrations before mixing.

Manual mixing experiments with fluorescence detection were performed on a Spex Fluorolog 2 fluorometer with excitation at 329 nm and emission at 397 nm. Dead time due to hand mixing was 15 s. Other experimental conditions were the same as for stopped-flow experiments. Kinetics for ^{32}P-labeled substrate were measured by gel methods with autoradiography detection as described by Bevilacqua et al. (1994).

Optical Melting. Melting of L-21 *Sca*I was followed on a Gilford 250 spectrophotometer at both 260 and 280 nm. L-21 *Sca*I was dried in a Speed-Vac, dissolved in the appropriate buffer, and incubated at 50 °C for 10 min and then at room temperature for at least 10 min before the optical cell was filled. The rate of heating was 0.5 °C/min.

RESULTS

UV Melting Curves Indicate That Global Tertiary Folding Is Complete at 20 °C When $Mg^{2+} \geq 2$ mM. Mg^{2+} concentration can affect tertiary interactions that give rise to the global folding of the ribozyme (Inoue & Cech, 1985; Latham & Cech, 1989; Grosshans & Cech, 1989; Jaeger et al., 1990; Celander & Cech, 1991; Wang & Cech, 1994; Zarrinker & Williamson, 1994), as well as local foldings (Murphy & Cech, 1993). A chemical mapping and optical melting study at 10 mM Mg^{2+} showed that the lowest temperature transition in the melting of L-21 *Sca*I is associated with cooperative melting of the global tertiary structure (Banerjee et al., 1993). A site-directed mutagenesis and optical melting study of the sunY group I ribozyme came to the same conclusion (Jaeger et al., 1993). Figure 1 shows optical melting curves and their derivatives for L-21 *Sca*I at 260 nm for 1, 2, and 5 mM Mg^{2+}. Similar curves are observed at 280 nm (data not shown). The differentiated curves all show three transitions above 50 °C. At 1 mM Mg^{2+}, however, the transition at about 53 °C is relatively broad, and the derivative curve at low temperature never goes to zero, even at 10 °C, suggesting the transition is not complete. The curves at 2 and 5 mM Mg^{2+}, however, exhibit a cooperative transition between 50 and 60 °C that is similar to that associated with tertiary structure formation at 10 mM Mg^{2+} (Banerjee et al., 1993), and that is complete by 20 °C. To avoid effects due to formation of global tertiary structure at $Mg^{2+} \geq 2$ mM, the experiments reported below were done at ≤20 °C, where global folding appears complete.

Rate of PyrCUCUA Base-Pairing with Ribozyme Increases between 5 and 10 mM Mg^{2+} but Rate of Dissociation Does Not. The short substrate pyrCUCUA, denoted as s, provides a convenient system for measuring rates of association and dissociation of the P1 helix, because it does not dock into the catalytic core in the absence of prG, and the rate of dissociation is fast enough to provide a reliable measurement by stopped-flow (Bevilacqua et al., 1994). Thus it binds to form P1 in a simple bimolecular reaction:

$$s + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} s{\cdot}R \quad (2)$$

Rate constants for formation of the P1 helix by pyrCUCUA have been measured previously at 5 mM Mg^{2+}, pH 7.5, 15 °C (Bevilacqua et al., 1994). To determine whether Mg^{2+} concentration affects these rate constants for base pairing, rates of formation for the pyrCUCUA·L-21 *Sca*I complex were measured at 10 mM Mg^{2+}, pH 7.5, 15 °C, by rapidly mixing pyrCUCUA with L-21 *Sca*I. Single-exponential

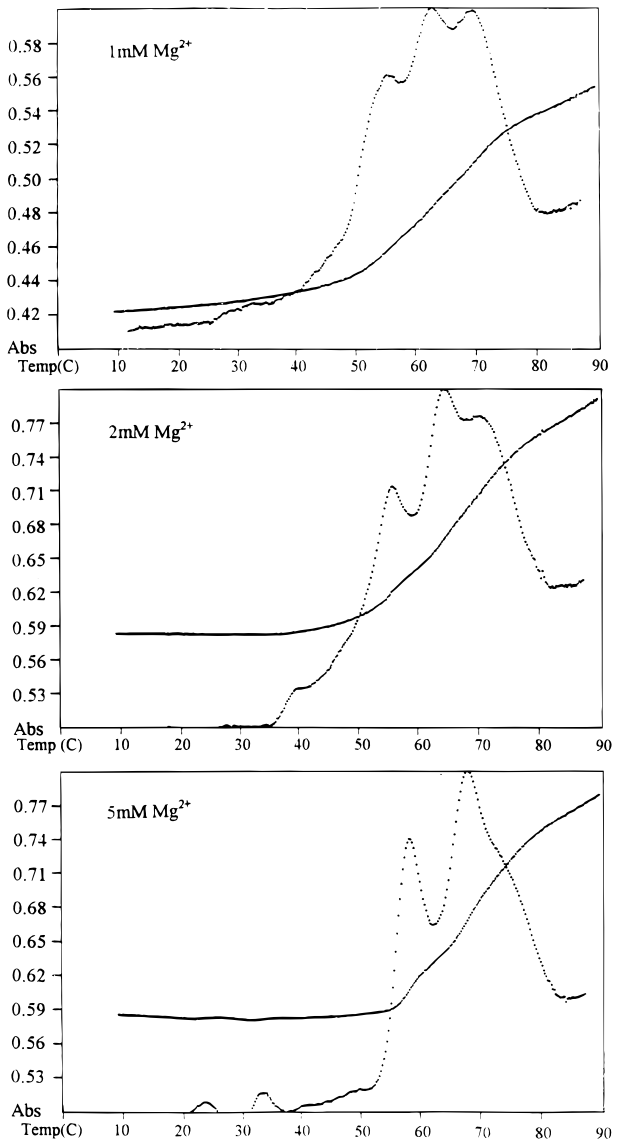

FIGURE 1: UV melting curves at 260 nm and their derivatives (dotted lines) for L-21 *Sca*I ribozyme. Buffer is 160 mM Na^+, pH = 6.5, with (top to bottom panels) 1, 2, or 5 mM Mg^{2+}.

fluorescence transients were observed. For a simple bimolecular association with pyrCUCUA in excess, the observed rate constant, τ^{-1}, is given by

$$\tau^{-1} = k_1[\text{pyrCUCUA}] + k_{-1} \qquad (3)$$

where k_1 and k_{-1} are the on and off rate constants, respectively, for the bimolecular association. Figure 2 shows a plot of the observed rate constants as a function of pyrCUCUA concentration. A linear fit to this plot gives $k_1 = (8.7 \pm 0.7) \times 10^6\ M^{-1}\ s^{-1}$ and $k_{-1} = 25 \pm 4\ s^{-1}$ at 10 mM Mg^{2+}. These can be compared with the reported values of $(2.7 \pm 0.7) \times 10^6\ M^{-1}\ s^{-1}$ and $17 \pm 5\ s^{-1}$ measured at 5 mM Mg^{2+} (Bevilacqua et al., 1994). Evidently, the concentration of Mg^{2+} affects the on rate but not the off rate for base pairing.

Observed Rate Constants for Docking and a Slower Fluorescence Transient Have Different Dependences on Mg^{2+} Concentration. It has previously been shown at 15 °C, 5 mM Mg^{2+}, in the absence of prG cofactor, that pyrCCUCUA binds to L-21 *Sca*I by base pairing to the internal guide sequence but does not dock into the catalytic site (Bevilacqua et al., 1994). Docking of substrate can then

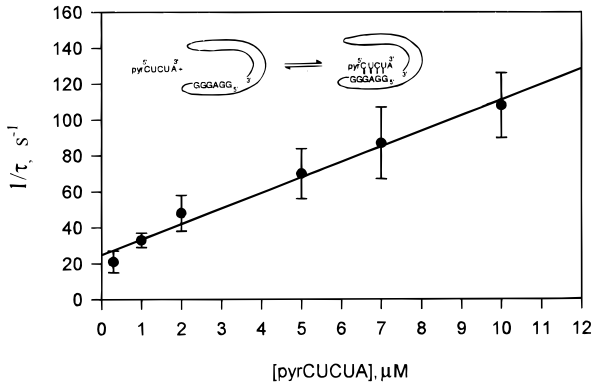

FIGURE 2: Dependence of observed rate constant on pyrCUCUA concentration for binding to 50 nM L-21 *Sca*I at 15 °C. Data points come from fits of fluorescence versus time to a single exponential, truncated at 6 half-lives. Error bars represent standard deviation in observed rate constants. Buffer is 160 mM Na^+, pH = 7.5, with 10 mM Mg^{2+}.

be induced by binding prG (Bevilacqua et al., 1994). To measure the dependence of the docking and subsequent steps on Mg^{2+} concentration, preformed pyrCCUCUA·L-21 *Sca*I complex was rapidly mixed in a stopped-flow apparatus with an equal volume of 10 mM prG. Typical traces of fluorescence intensity vs time at pH 6.5 and 20 °C are shown in Figure 3. For most concentrations of Mg^{2+}, the traces are fit well by a double exponential. The faster exponential is presumably due to docking of pyrCCUCUA into the catalytic site (Bevilacqua et al., 1992, 1994). From 4 to 6 mM Mg^{2+}, the double-exponential fits are not as good as at other concentrations of Mg^{2+} (see Figure 3). Thus these traces were fit with three exponentials (fit not shown). Figure 4 shows plots of averages of observed rate constants as a function of Mg^{2+} concentration. Open and closed symbols in Figure 4 denote transients having negative and positive amplitudes, respectively, i.e., fluorescence quenching or enhancement. Quenching is observed at 2 mM Mg^{2+}, whereas enhancement is observed at $[Mg^{2+}] \geq 4$ mM. Evidently, prG binds at all Mg^{2+} concentrations studied, but positioning of the pyrCCUCUA substrate induced by prG binding is different at low and high $[Mg^{2+}]$. The traces in Figure 3 also show that, for 4 mM $\leq [Mg^{2+}] \leq$ 6 mM, the two slower observed rate constants are associated with a larger total amplitude than the fastest observed rate constant (τ_1^{-1}), whereas the reverse is true for $[Mg^{2+}] \geq 7$ mM. Rates of the slowest fluorescence transient were also measured by manual mixing in a fluorometer and are consistent with those measured by stopped-flow spectroscopy. At 5 mM Mg^{2+}, rates were also measured as a function of prG concentration and were the same within experimental error from 1 to 5 mM prG (data not shown).

The rate of the transesterification reaction can be measured by gel methods with autoradiography detection using ^{32}P-labeled substrate. Figure 4 shows the observed rate constants at pH 6.5, 20 °C, as a function of $[Mg^{2+}]$. The observed rate constants measured directly for cleavage are similar to those of the slowest fluorescence transient, consistent with the slowest transient reporting the rate of transesterification. This is consistent with previous observations at 5 mM Mg^{2+}, 15 °C (Bevilacqua et al., 1994). The ^{32}P assay also detects a very slow transesterification at 2 mM Mg^{2+}, where fluorescence quenching rather than enhancement is observed. Thus, at 2 mM Mg^{2+}, most of the substrate may be bound in an unproductive mode that quenches fluorescence. This

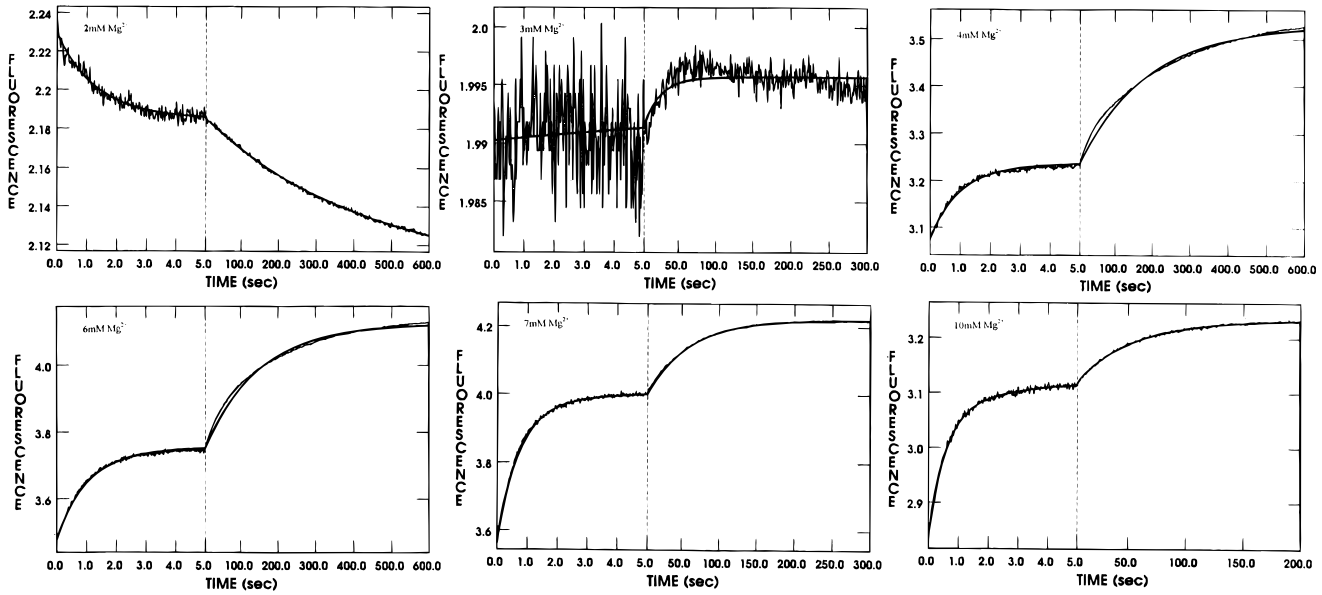

FIGURE 3: Representative traces for dependence of fluorescence intensity on time after equal volumes of pyrCCUCUA·L-21 *Sca*I and prG were mixed at 20 °C. Final concentrations after mixing are 0.15 μM ribozyme, 0.3 μM pyrCCUCUA, and 5 mM prG. Data were fit to $F = F_\infty + F^{-t/\tau_1} + F^{-t/\tau_2}$, and the fitted curve is also shown. For 4−6 mM Mg^{2+}, data were also fit to $F = F_\infty + F^{-t/\tau_1} + F^{-t/\tau_2} + F^{-t/\tau_3}$; the fitted curve is not shown but is essentially coincident with experimental points. Buffer is 160 mM Na^+, pH = 6.5, with indicated [Mg^{2+}].

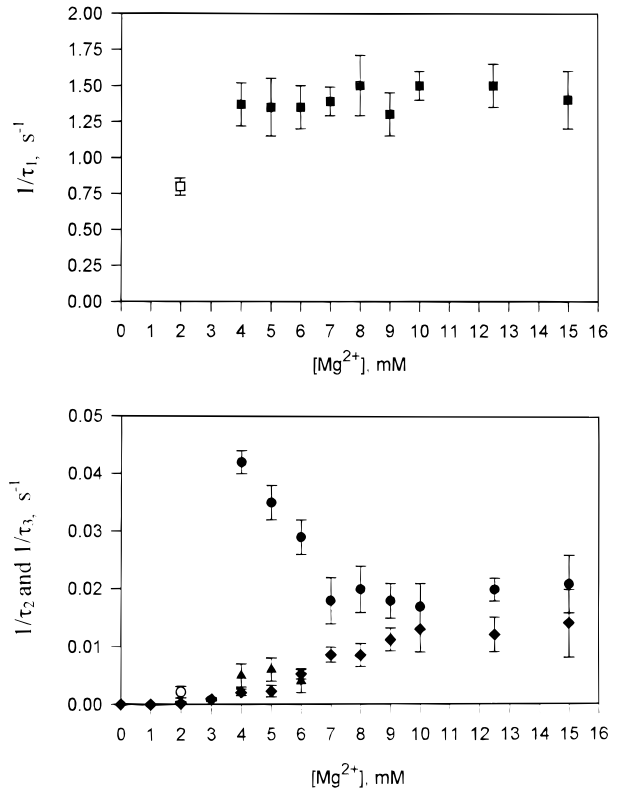

FIGURE 4: (Top) Plot of observed rate constant of fastest fluorescence transient (□, ■), τ_1^{-1}, as a function of Mg^{2+} concentration. (bottom) Plot of observed rate constants, τ_2^{-1} and τ_3^{-1} (○, ●, ▲), from fluorescence transients, and of observed first-order rate constant measured by gel methods with autoradiography detection for cleavage of ^{32}P-labeled pyrCCUCUA (◆) (0.15 μM L-21 *Sca*I, 0.30 μM pyrCCUCUA, and 5 mM prG) as a function of Mg^{2+} concentration at 20 °C. □ and ○ denote fluorescence quenching. ■, ●, and ▲ denote fluorescence enhancement. Fluorescence data were fit to three exponentials when 4 mM ≤ [Mg^{2+}] ≤ 6 mM and to two exponentials at other [Mg^{2+}]. Data for ^{32}P cleavage were fit to single exponentials. No product burst was observed. Buffer is 160 mM Na^+, pH = 6.5. Error bars represent standard deviation in rates measured on different days.

preequilibrium, however, may be followed by an unfavorable conformational change that leads to reaction. This would result in the slow overall rate of transesterification observed with ^{32}P.

Rates were also measured by fluorescence-detected stopped-flow spectroscopy at pH 7.5 and 20 °C for selected Mg^{2+} concentrations. These traces were all fit well by a double exponential (see typical traces in supporting information; see paragraph at end of paper regarding supporting information). Figure 5 shows the observed rate constants as a function of Mg^{2+}. The observed rate constants for the faster transient are similar to those at pH 6.5, indicating docking is relatively insensitive to pH in this range. The observed rate constants for the slowest transient, however, are roughly a factor of 5−20 faster at pH 7.5 compared with pH 6.5. This is within experimental error of the factor of 10 expected for the transesterification step (Herschlag et al., 1993b; Herschlag & Khosla 1994), since day-to-day fluctuations in this rate can be as large as 2-fold (Herschlag & Cech, 1990). At both pH 6.5 and 7.5, the observed rate constant of the faster fluorescence transient is essentially constant when [Mg^{2+}] ≥ 4 mM, whereas the observed rate constant of the slower transient increases between 4 and 8 mM Mg^{2+} (see Figures 4 and 5). Evidently, the two processes depend on Mg^{2+} in different ways.

When [Mg^{2+}] + [Ca^{2+}] = 15 mM, Mg^{2+} Dependences of Observed Rate Constants for Docking and the Slower Fluorescence Transient Differ from Those Observed in the Absence of Ca^{2+}. Fluorescence-detected stopped-flow spectroscopy was also used to measure rates as a function of Mg^{2+} concentration when the total concentration of divalent cation was held constant at 15 mM with Ca^{2+}. Figure 6 shows typical traces at pH 7.5, 20 °C, and Figure 7 shows observed rate constants vs [Mg^{2+}]. When [Mg^{2+}] + [Ca^{2+}] = 10 mM at 15 °C, a similar observed rate constant vs [Mg^{2+}] trend is observed (see supporting information). At 2 and 3 mM Mg^{2+}, the fluorescence transients are fit well by a single exponential, whereas a double exponential is required when [Mg^{2+}] ≥ 4 mM. Evidently, when [Mg^{2+}] + [Ca^{2+}] = 15 mM, at least 4 mM Mg^{2+} is required to support the step following docking. This is consistent with previous studies showing no transesterification in the pres-

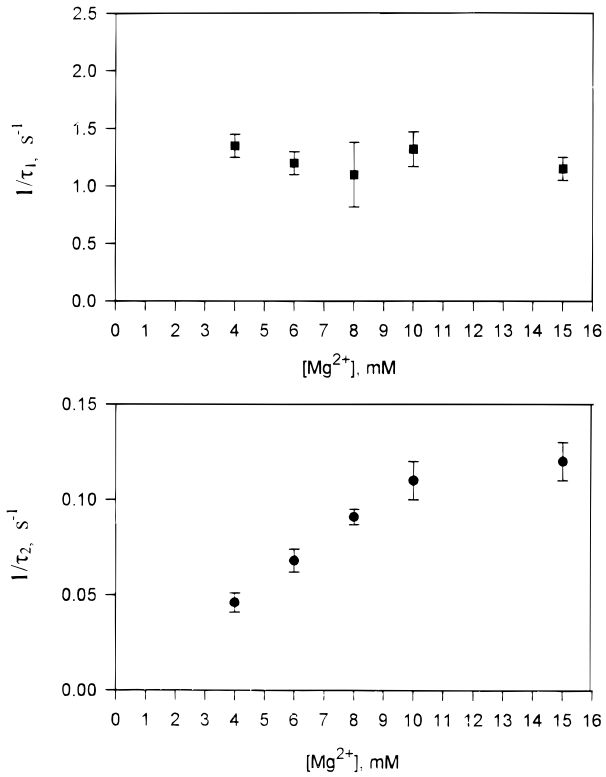

FIGURE 5: (Top) Plot of observed rate constant of fastest fluorescence enhancement, τ_1^{-1}, as a function of Mg^{2+} concentration. (Bottom) Plot of observed rate constant of slower fluorescence enhancement, τ_2^{-1}, as a function of Mg^{2+} concentration at 20 °C. Buffer is 160 mM Na^+, pH = 7.5. Error bars represent standard deviation of observed rate constants measured on different days. Fluorescence enhancement was also observed at 3 mM Mg^{2+}, but the observed rate constant was variable. Fluorescence quenching was observed at 1 and 2 mM Mg^{2+}.

ence of only Ca^{2+} (Grosshans & Cech, 1989; Wang & Cech, 1994).

In the presence of Ca^{2+}, the observed rate constants of both the faster and slower transients increase roughly 2-fold from 7 to 11 mM Mg^{2+}. This transition is not observed in the absence of Ca^{2+} (compare Figures 5 and 7).

Binding of pdG Induces a Conformational Change That Does Not Lead to Transesterification. Figure 8 shows fluorescence transients observed at 3 and 10 mM Mg^{2+} upon mixing preformed pyrCCUCUA·L-21 *Sca*I complex with an equal volume of 10 mM pdG. In both cases, two transients are observed, with the amplitude of the faster transient much larger than for the slower one. The slower transient was not observed in a hand mixing experiment in a fluorometer, so that it may arise from an artifact in the stopped-flow apparatus at long times. The results show that pdG binds in the presence of 3 and 10 mM Mg^{2+} and induces a fluorescence quenching similar to that observed for binding of prG at 2 mM Mg^{2+}. The observed rate constants for both pdG and prG binding in the presence of all Mg^{2+} concentrations studied are about 1 s^{-1}. This suggests that the binding induces a similar conformational change but that the final positioning of pyrCCUCUA is different for prG at $[Mg^{2+}] \geq 4$ mM when compared with either pdG at 3 or 10 mM Mg^{2+} or prG at 2 mM Mg^{2+}.

Reactivity of pdG was tested by incubating 5 mM pdG with 150 nM L-21 *Sca*I and ^{32}P-labeled pyrCCUCUA for up to 48 h at pH 6.5 in the presence of 5 mM Mg^{2+}. No reaction was observed when the reaction mixture was run on a 20% acrylamide/8 M urea denaturing gel.

DISCUSSION

Metal ions are known to be important for both folding and catalysis by RNA (Grosshans & Cech, 1989; Jaeger et al., 1990; Cech, 1990; Dahm & Uhlenbeck, 1991; Piccirrilli et al., 1993; Smith & Pace, 1993; Beebe et al., 1996; Cate et al., 1996; Cate & Doudna, 1996; Zarrinker & Williamson, 1994; Pan et al., 1993; Laing et al., 1994; Lu & Draper, 1994; Pyle, 1993; Bassi et al., 1995). The group I ribozyme provides a convenient system for studying RNA folding and catalysis because it does not require protein for activity and because substrates can be added *in trans* (Zaug et al., 1988; Inoue & Kay, 1987; Herschlag & Cech, 1990). Transient kinetic studies with spectroscopic probes provide an opportunity to study individual steps required for both folding and catalysis by this ribozyme (Turner et al., 1996). These methods also permit the study of steps that are too fast for conventional measurements (Johnson, 1986, 1992; Fierke & Hammes, 1995; Turner, 1986; Bernasconi, 1976). Thus both pyrene- (Bevilacqua et al., 1992, 1993, 1994; Kierzek et al., 1993) and ϵA- (Turner et al., 1996) labeled substrates have been used to follow steps in the catalytic mechanism. One of these steps, docking of substrate into the catalytic core, is associated with a favorable entropy change, suggesting it may be coupled with uptake of Mg^{2+} ions (Li et al., 1995; Narlikar & Herschlag, 1996). Here we use the stopped-flow method with fluorescence detection to determine the rates of docking and other steps in the catalytic mechanism as a function of Mg^{2+} concentration.

Global Folding, Docking of Substrate, and the Step Following Docking Have Different Dependences on [Mg^{2+}], and All These Transitions Are Dependent on Mg^{2+} in a Cooperative Manner. The shapes of the UV melting curves shown in Figure 1 suggest that at ≤20 °C, the majority of tertiary interactions required for global folding of L-21 *Sca*I are formed when $Mg^{2+} \geq 2$ mM. This is consistent with previous chemical mapping and activity studies at 42 °C (Latham & Cech, 1989; Wang & Cech, 1994) and 37 °C (Zarrinker & Williamson, 1994). As shown in Figures 3 and 4, however, the fastest fluorescence transient is associated with fluorescence quenching at 2 mM Mg^{2+}, whereas it is associated with fluorescence enhancement when $[Mg^{2+}] \geq 4$ mM. Evidently, there is a local Mg^{2+}-dependent transition required for productive docking that is not revealed by UV melting studies. This transition is very cooperative since it occurs over a 2 mM range in Mg^{2+} concentration. From 4 to 15 mM Mg^{2+}, the observed rate constant for docking is essentially constant (Figures 4 and 5). In contrast, the observed rate constants of the slower fluorescence transients (Figures 4 and 5) and of transesterification as measured with a ^{32}P assay (Figure 4) continue to change above 4 mM Mg^{2+}. This suggests a second local transition that is dependent on Mg^{2+} concentration in the narrow range of 4−10 mM Mg^{2+}, again suggesting cooperativity.

Folding Required for Docking, but Not the Slower Transient, May Be Supported by Ca^{2+} Alone. At 15 mM Ca^{2+} in the absence of Mg^{2+}, a single-exponential fluorescence enhancement with small amplitude is observed (Figure 6). As Ca^{2+} is replaced with Mg^{2+}, the rate of this enhancement is constant up to 6 mM Mg^{2+} (Figure 7). The amplitude of the enhancement, however, is considerably larger in the presence than in the absence of Mg^{2+}. This suggests Ca^{2+} alone may at least partially induce the local folding required to give docking with a fluorescence

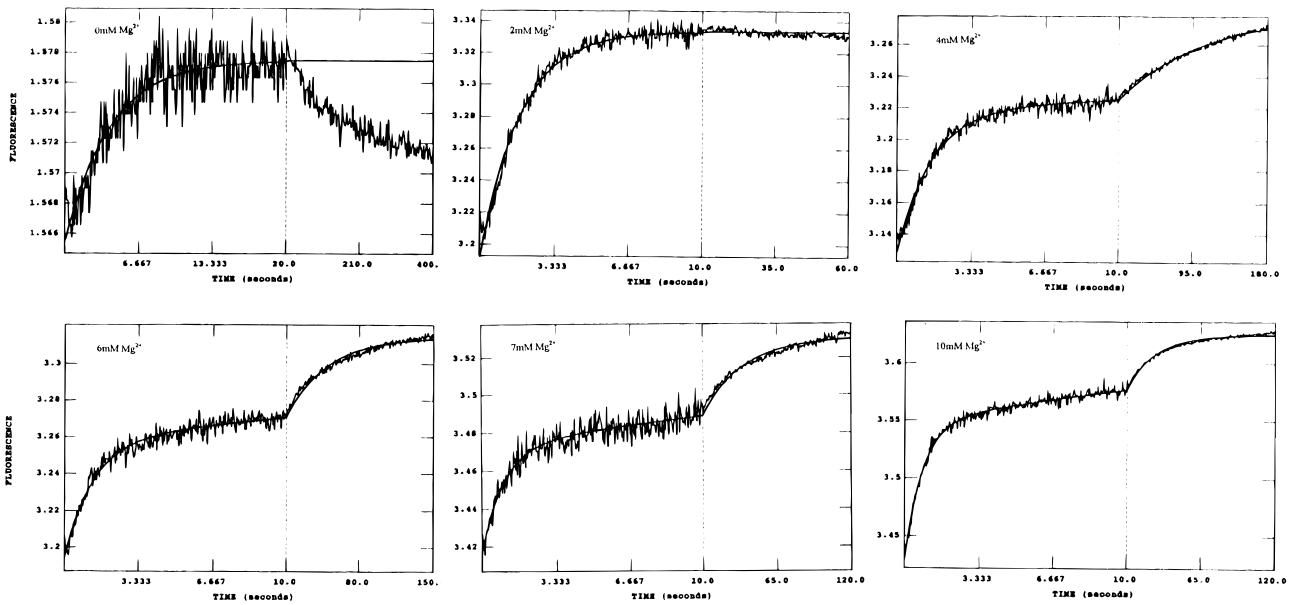

FIGURE 6: Representative traces for dependence of fluorescence intensity on time after equal volumes of pyrCCUCUA·L-21 *Sca*I and prG were mixed at 20 °C. Final concentrations after mixing are 0.14 μM ribozyme, 1 μM pyrCCUCUA, and 5 mM prG. Data are fit to $F = F_\infty + F^{-t/\tau_1} + F^{-t/\tau_2}$. Buffer is 160 mM Na^+, pH = 7.5, with indicated [Mg^{2+}], and total concentration of $Ca^{2+} + Mg^{2+} = 15$ mM.

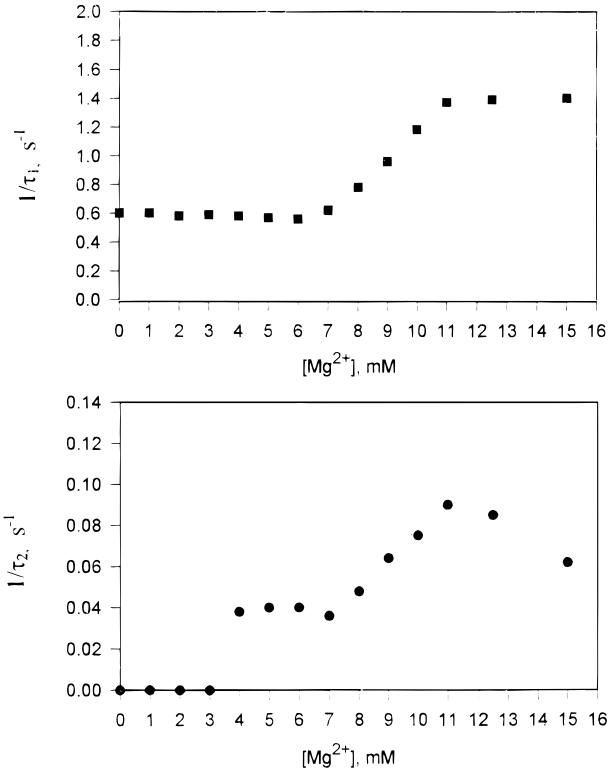

FIGURE 7: (Top) Plot of observed rate constant of fastest fluorescence transient, τ_1^{-1}, as a function of Mg^{2+} concentration. (bottom) Plot of observed rate constant of slower fluorescence transient, τ_2^{-1}, as a function of Mg^{2+} concentration. Buffer is 160 mM Na^+, pH = 7.5, and total concentration of $Ca^{2+} + Mg^{2+} = 15$ mM.

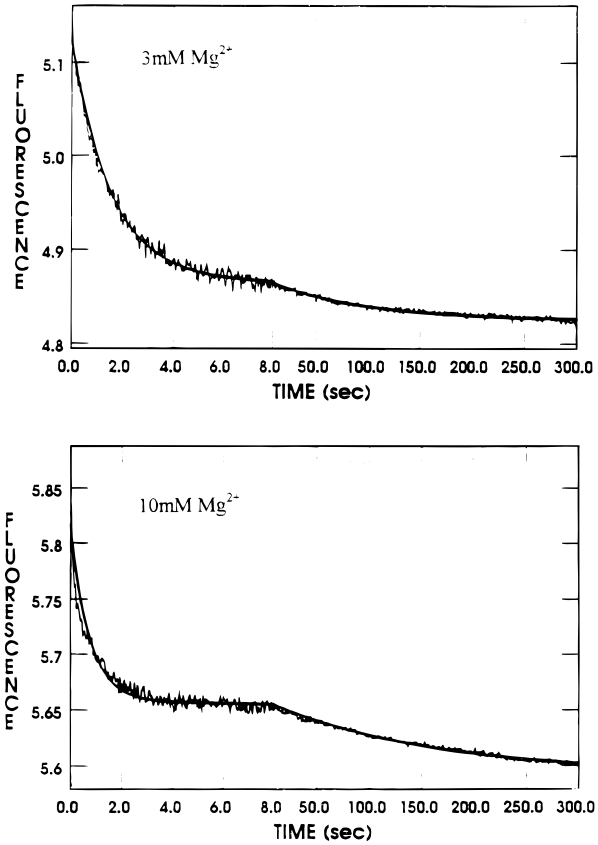

FIGURE 8: Representative traces for dependence of fluorescence intensity on time after equal volumes of pyrCCUCUA·L-21 *Sca*I and pdG were mixed at 20 °C. Final concentrations after mixing are 0.15 μM ribozyme, 0.3 μM pyrCCUCUA, and 5 mM pdG. Buffer is 160 mM Na^+, pH = 6.5, with 3 (top) or 10 (bottom) mM Mg^{2+}. Observed rate constants for the faster transient are 0.65 and 1.4 s^{-1} for 3 and 10 mM Mg^{2+}, respectively. Similar results were observed from 1 to 15 mM Mg^{2+}.

enhancement. Ca^{2+} alone, however, does not induce the local folding required for the slower fluorescence enhancements observed only when [Mg^{2+}] ≥ 4 mM. This provides additional evidence that the local folding domains required for docking and the slower fluorescence transient are different. In the presence of Ca^{2+}, the transition from one to two fluorescence enhancements occurs between 3 and 4 mM Mg^{2+}, providing confirmation that this local transition is cooperative.

When [Ca^{2+}] + [Mg^{2+}] = 15 mM, Observed Rate Constants of both Docking and the Slower Fluorescence Transient Increase as Mg^{2+} Increases from 7 to 11 mM, Suggesting Another Cooperative Transition. When [Ca^{2+}] + [Mg^{2+}] = 15 mM, the observed rate constants of docking

and the slower fluorescence transient are constant from 4 to 7 mM Mg^{2+}. From 7 to 11 mM Mg^{2+}, however, both observed rate constants increase (Figure 7), suggesting a set of cation binding sites that can be filled by either Ca^{2+} or Mg^{2+}. Evidently, however, Mg^{2+} facilitates both docking and the second conformational change more than Ca^{2+}. A Hill plot (Wyman & Gill, 1990; Fersht, 1985) of this data provides a Hill constant of 8.7 ± 1.3, indicating that this transition is also cooperative, involving uptake of seven or more Mg^{2+} ions.

The above results can be compared with photo-cross-linking results at 42 °C for binding of GGCCCUCU to L-21 *Sca*I functionalized at the 5′ end with azidophenacyl (Wang & Cech, 1994). Cross-linking to the active site was reduced 92% when 10 mM Mg^{2+} was replaced with 10 mM Ca^{2+}. This is consistent with the small amplitude observed for the fastest fluorescence transient at 15 mM Ca^{2+} in the absence of Mg^{2+} (Figure 6). Wang and Cech (1994) also observed no reactivity for the substrate GGCCCUCUAAAAA in the presence of 10 mM Ca^{2+} without Mg^{2+}, even when the ribozyme was pre-cross-linked in the presence of Mg^{2+} to facilitate docking of substrate to the catalytic site. This is consistent with the absence of a second fluorescence enhancement at 15 mM Ca^{2+} in the absence of Mg^{2+} (Figure 6). The Mg^{2+} dependence of the photo-cross-linking results showed a single two-state transition at about 2 mM Mg^{2+}, consistent with the changes in melting curves as a function of [Mg^{2+}] shown in Figure 1. This transition probably reflects formation of global tertiary structure. An X-ray "footprinting" study of L-21 *Sca*I folding in the presence of 10 mM Na^+ at 42 °C has revealed multiple transitions with different Hill constants at about 0.5 mM Mg^{2+} (Sclavi et al., 1997). The transitions reported here at >2 mM Mg^{2+}, however, appear to report more local formation of ribozyme structure.

Folding for Reactive Docking of PyrCCUCUA Requires the 2′ OH of prG. Transesterification involving a group I intron or ribozyme has never been observed with a dG substrate (Bass & Cech, 1986; Moran et al., 1993), and these results are confirmed here. Moreover, gel mobility studies detect a conformational change induced by prG but not pdG in a construct containing partial exons and the *Tetrahymena* group I intron (Emerick & Woodson, 1994). Thus it is of interest to determine the role of the 2′ OH group of prG for various steps in the reaction mechanism. When prG is mixed with preformed pyrCCUCUA·L-21*Sca*I complex at [Mg^{2+}] ≥ 4 mM, a fluorescence enhancement is observed that presumably reports docking of substrate into the catalytic site. In contrast, mixing with pdG in the presence of 1−15 mM Mg^{2+} results in fluorescence quenching (Figure 8). Evidently, the 2′ OH group of prG provides an interaction important for productive docking of substrate, and the lack of this interaction cannot be overcome by up to 15 mM Mg^{2+}. The results are consistent with a proposal that the 2′ OH of prG can help align the splice site by forming a hydrogen bond to a nonbridging oxygen of the reactive phosphate (Turner et al., 1996). Such an interaction would be similar to that observed in the exonuclease site of Klenow fragment, where an OH group from tyrosine helps position the reactive phosphate (Freemont et al., 1988; Beese & Steitz, 1991). Similar tyrosine hydroxyl to phosphate hydrogen bonds have been observed in other protein−nucleic acid complexes (Valegard et al., 1994; LeCuyer et al., 1996; Foster et al., 1997). Alternatively, the 2′ OH could have an indirect effect on substrate positioning. For example, it could help position a Mg^{2+} ion that is important for proper docking (Sugimoto et al., 1988, 1989; Sjögren et al., 1997). A 2′ OH interaction could also help account for the cooperative binding observed between oligopyrimidine substrates and prG by McConnell et al. (1993). This interaction may also account for the anticooperative binding of pyrCUCU and prG (Bevilacqua et al., 1993), since the absence of the reactive phosphate could result in the hydrogen-bonding potential of the desolvated 2′ OH being unsatisfied.

There May Be an Additional Step besides Docking and Phosphoryltransfer. There are several observations suggesting a conformational change in addition to docking before transesterification occurs. At pH 6.5, traces are not fit well by a double exponential when 4 mM ≤ [Mg^{2+}] ≤ 6 mM (Figure 3). This has also been observed at 15 °C with 5 mM Mg^{2+} (Bevilacqua et al., 1994). In addition, the total amplitude of the slowest fluorescence transients is larger than that of the fastest one when 4 mM ≤ [Mg^{2+}] ≤ 6 mM but smaller than the fastest one when [Mg^{2+}] ≥ 7 mM. This is consistent with a model in which there is an additional step with a rate slower than docking when [Mg^{2+}] ≤ 6 mM, but faster than docking when [Mg^{2+}] ≥ 7 mM. In this model, the amplitude associated with this step is associated with the slowest transients below 7 mM Mg^{2+} and with the faster transient when [Mg^{2+}] ≥ 7 mM. This model is also consistent with the observation that the rate of the slowest fluorescence transient in a three-exponential fit is similar to the rate of transesterification as measured by a ^{32}P assay when 4 mM ≤ [Mg^{2+}] ≤ 6 mM. Thus the mechanism for transesterification may involve more than initial docking followed by cleavage. That is, the data are consistent with the following minimal mechanism:

$$S{\cdot}R + prG \rightleftharpoons S{\cdot}R{\cdot}prG \rightleftharpoons [S{\cdot}R{\cdot}prG]' \rightleftharpoons [S{\cdot}R{\cdot}prG]'' \rightleftharpoons P{\cdot}R{\cdot}prGA \quad (4)$$

Here S·R·prG, [S·R·prG]′, and [S·R·prG]″ are the undocked, docked, and rearranged ternary complexes, respectively. Alternatively, the intermediate rate could be associated with refolding of a subpopulation of ribozyme to the structure required for docking.

Mg^{2+} Dependence of Rate Constants for the Base-Pairing Step Is Similar to That Observed for Duplex Formation by a Short Oligonucleotide. It is known that Mg^{2+} can favor duplex formation by oligonucleotides (Williams et al., 1989). The effects of Mg^{2+} on duplex formation between oligonucleotides and large RNAs has not been studied, however. Oligonucleotides are increasingly being used to target large RNAs (Agrawal et al., 1989; Sarver et al., 1990; Matsukura et al., 1991; Ojwang et al., 1992; Koizumi et al.,1992; Cantor et al., 1993), so that the salt dependence of such interactions is of interest. The results presented here show that increasing Mg^{2+} concentration from 5 to 10 mM in the presence of 160 mM Na^+ increases the bimolecular rate constant for pyrCUCUA binding to L-21 *Sca*I by about 3-fold, whereas the rate constant for dissociation is essentially unchanged. This is similar to the results of Williams et al. (1989) for $(dGCATGC)_2$, where increasing Mg^{2+} concentration from 2 to 10 mM in the presence of 12 mM Na^+ increased the bimolecular rate constant for association 3-fold while leaving the rate constant for dissociation unchanged. This behavior is expected from Manning's model of association in which counterion condensation occurs before base-pair formation and hence before the rate-limiting step for duplex formation

(Manning, 1976, 1978; Williams et al., 1989).

SUMMARY AND IMPLICATIONS

Transient kinetic studies with spectroscopic detection permit monitoring of individual steps in ribozyme folding and catalysis. The results presented here indicate at least four and possibly five classes of specific cation binding sites are important for folding the group I intron into an active ribozyme. One class of sites is required for global folding as observed previously (Grosshans & Cech, 1989; Celander & Cech, 1991; Wang & Cech, 1994). A second class is required for docking of substrate into the catalytic core. These sites probably can be filled by either Ca^{2+} or Mg^{2+}, although Mg^{2+} is preferred. A third class of sites is required for transesterification and cannot be filled by Ca^{2+}, as observed previously (Grosshans & Cech, 1989). A fourth class of sites can be filled by either Ca^{2+} or Mg^{2+}, but Mg^{2+} enhances the observed rate constants for docking and a slower fluorescence transient by about 2-fold over Ca^{2+}. There is also evidence of Mg^{2+} dependence for a third fluorescence transient with a rate intermediate between docking and transesterification when 4 mM $\leq$ [Mg^{2+}] $\leq$ 6 mM. All these transitions are promoted by Mg^{2+} in a highly cooperative manner, suggesting many Mg^{2+} ions are involved in each transition. Cate et al. (1996) have recently identified an A-rich bulge that binds 2 Mg^{2+} ions in the crystal structure of the P4−P6 domain of the L-21 *Sca*I ribozyme. This bulge forms a crucial tertiary interaction, suggesting that Mg^{2+} cores may be important structures for folding RNA (Cate et al., 1997). The docking of helices could also be associated with formation of a group of Mg^{2+} binding sites (Li et al., 1995). The kinetic results reported here are consistent with this idea and suggest that several such domains must form for maximum ribozyme activity. These domains may form prior to, coincident with, or after substrate docking. Thus transient kinetics experiments reveal a complex set of Mg^{2+}-dependent domain foldings that are required for optimal activity. This probably represents a fundamental difference between ribozymes and protein enzymes. Domains in the polyanionic ribozymes require many divalent cations to fold into the condensed structures required for catalysis.

The cooperative nature of the transitions suggests that it may be possible to use chemical mapping as a function of Mg^{2+} concentration to identify the local tertiary interactions responsible for facilitating various steps in the catalytic mechanism (Murphy & Cech, 1993). For example, at 20 °C and pH 6.5, global folding appears complete at 2 mM Mg^{2+}, but docking associated with a fluorescence enhancement is not observed until 3 mM Mg^{2+}. Similarly, the slowest transient is barely detectable at 3 mM Mg^{2+} but is saturated at 10 mM Mg^{2+}. The effects of site-directed mutagenesis on these various transitions could provide further insights. Thus this work provides a foundation for future structure−function studies. Such studies may also be able to identify long-range tertiary interactions useful for modeling this and other RNAs.

The results presented here on the rates of pyrCUCUA base pairing at 5 and 10 mM Mg^{2+} are consistent with nonspecific binding of Mg^{2+} to duplexes as suggested by Manning (1976, 1978). Thus a thorough understanding of RNA folding will require insight into many different types of interactions of Mg^{2+} with RNA.

The 2′ OH of prG is required for productive docking of pyrCCUCUA substrate. This is consistent with models in which this 2′ OH group makes direct or indirect contacts with a nonbridging oxygen at the reactive site on the substrate (Turner et al., 1996; Sugimoto et al., 1988, 1989; Sjögren et al., 1997) and provides another example of the importance of 2′ OH groups in RNA folding and function (Bass & Cech, 1986; Sugimoto et al., 1989; Pyle & Cech, 1991; Pyle et al., 1992; Bevilacqua & Turner, 1991; Dahm & Uhlenbeck 1991; Jaeger et al., 1993; Herschlag et al., 1993a,b; Pley et al., 1994; Cate et al., 1996; Abramovitz et al., 1996).

NOTE ADDED IN PROOF

In preliminary experiments at 5 mM Mg^{2+} and 5 mM prG, a switch from fluorescence enhancement to fluorescence quenching is observed when [Na^{+}] $\geq$ 270 mM.

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SUPPORTING INFORMATION AVAILABLE

Two figures, showing typical fluorescence traces at pH 7.5 and 20 °C and observed rate constants vs [Mg^{2+}] at 15 °C when ([Mg^{2+}] + [Ca^{2+}]) = 10 mM (2 pages). Ordering information is given on any current masthead page.

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