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REPORT

Dissection of a metal-ion-mediated conformational change in *Tetrahymena* ribozyme catalysis

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ABSTRACT

Conformational changes are often required for the biological function of RNA molecules. In the *Tetrahymena* group I ribozyme reaction, a conformational change has been suggested to occur upon binding of the oligonucleotide substrate (S) or the guanosine nucleophile (G), leading to stronger binding of the second substrate. Recent work showed that the two substrates are bridged by a metal ion that coordinates both the nonbridging reactive phosphoryl oxygen of S and the 2′-OH of G. These results suggest that the energy from the metal ion•substrate interactions is used to drive the proposed conformational change. In this work, we provide an experimental test for this model. The results provide strong support for the proposed conformational change and for a central role of the bridging metal ion in this change. The results from this work, combined with previous data, allow construction of a two-state model that quantitatively accounts for all of the observations in this and previous work. This model provides a conceptual and quantitative framework that will facilitate understanding and further probing of the energetic and structural features of this conformational change and its role in catalysis.

Keywords: conformational change; mechanistic analysis; metal ion; ribozyme; RNA catalysis

INTRODUCTION

Conformational changes are widespread in biological processes, playing crucial roles in the function of numerous RNA molecules (e.g., Costa et al., 1997; Lodmell & Dahlberg, 1997; Patel et al., 1997; Staley & Guthrie, 1998; Wilson & Noller, 1998; Doherty & Doudna, 2001 and references therein). To understand these conformational changes, it is necessary to delineate their thermodynamic and kinetic properties and to identify the molecular interactions that mediate these changes.

The *Tetrahymena* ribozyme (E) provides an attractive system for studying conformational changes in RNA function. This ribozyme, derived from a self-splicing group I intron, catalyzes a reaction analogous to the first step of self-splicing,

$$\underset{(S)}{CCCUCU_PA_5} + G_{OH} \rightarrow \underset{(P)}{CCCUCU_{OH}} + G_PA_5, \quad (1)$$

in which an exogenous guanosine nucleophile (G) cleaves a specific phosphodiester bond of an oligonucleotide substrate (S). The well-characterized catalytic activity of this ribozyme provides a sensitive assay that can be used to probe its structure and conformation (Herschlag & Cech, 1990). Extensive mechanistic work and mutational analyses have provided a detailed model for active site interactions that are crucial for catalysis (Fig. 1; Piccirilli et al., 1993; Weinstein et al., 1997; Strobel & Ortoleva-Donnelly, 1999; Shan & Herschlag, 1999; Shan et al., 1999a, 2001; Yoshida et al., 2000, and refs therein). The tools developed in these studies

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Abbreviations: E denotes the *Tetrahymena thermophila* L-21 *Sca*I ribozyme; S denotes the oligonucleotide substrate with the sequence $CCCUCUA_5$, without specification of modifications at the 2′-hydroxyl group (the individual oligonucleotide substrates used in this work are listed in Table 1); P denotes the oligonucleotide product with the sequence CCCUCU; G denotes the guanosine nucleophile, and G_N denotes the guanosine analog in which the 2′-hydroxyl of G is replaced by a 2′-amino group. M_C refers to the metal ion at site C (Fig. 1); Mg_C^{2+} and Mn_C^{2+} refers to the Mg^{2+} and Mn^{2+} ion bound at site C, respectively. $(E•S)_o$ and $(E•S)_c$ refer respectively to the open and closed complexes formed between the ribozyme and the oligonucleotide substrates (see text).

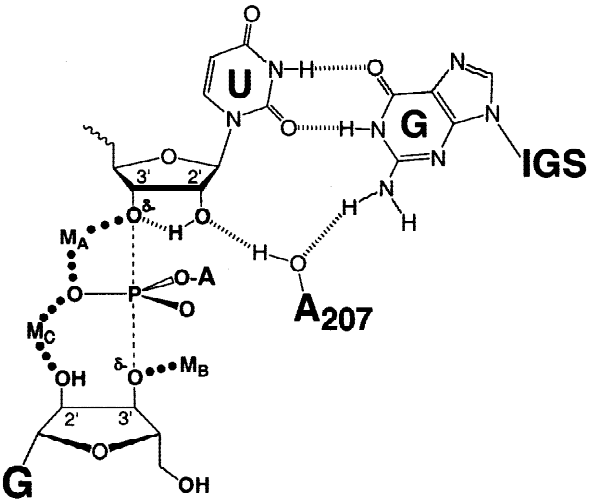


FIGURE 1. Model for catalytic interactions at the *Tetrahymena* ribozyme active site. The transition state of the reaction is shown, with the dashed lines (—) depicting the partial bonds from the reactive phosphorus to the leaving group and the incoming nucleophile, and δ- depicting the partial negative charges on the leaving group and the nucleophile. The dots (•) depict interactions of metal ions with their ligands, and thick dashed lines depict hydrogen bonding interactions. M_A, M_B, and M_C are the three previously identified catalytic metal ions at this RNA active site (Shan et al., 1999a). M_A coordinates the 3′-bridging oxygen of S and the *pro-*S_P oxygen of the reactive phosphoryl group (Piccirilli et al., 1993; Shan et al., 2001), M_B coordinates the 3′-oxygen of G (Weinstein et al., 1997), and M_C coordinates both the 2′-OH of G and the *pro-*S_P oxygen (Shan & Herschlag, 1999; Shan et al., 1999b, 2001). The 2′-OH of U(−1) donates a hydrogen bond to the neighboring 3′-bridging oxygen (Strobel & Ortoleva-Donnelly, 1999; Yoshida et al., 2000); there is evidence that this 2′-OH is part of a network of active site interactions that involves the 2′-OH of A_{207} and the exocyclic amine of the G•U pair that specifies the cleavage site (Strobel & Ortoleva-Donnelly, 1999).

allow us to detect structural changes of this ribozyme at the level of individual molecular interactions.

There is evidence for a conformational change within the active site upon binding of the oligonucleotide substrate and the guanosine nucleophile. The presence of bound S at the active site increases the affinity of the ribozyme for the guanosine nucleophile, and vice versa (McConnell et al., 1993; Bevilacqua et al., 1994). Further, this coupling effect is dependent on temperature: S and G enhance the binding of one another only above 10 °C, whereas at lower temperature, the guanosine nucleophile can bind as strongly to the free ribozyme as to the E•S complex even when S is not bound (McConnell & Cech, 1995). This strong substrate binding in the absence of the other substrate at low temperature suggested that coupling does not arise from a direct interaction between the two substrates. Rather, binding of S or G provides the energy for the change of the ribozyme to a conformation in which active site groups are better aligned to interact with the other substrate. Below 10 °C, this conformation is apparently adopted by the ribozyme without the free energy from substrate binding, thereby allowing strong binding of S or G even in the absence of the other substrate (McConnell & Cech, 1995).

What are the molecular interactions involved in this conformational change? The first clue came from the observation that the presence of bound oligonucleotide product (P) does not increase the affinity for G, even though P is more stably bound at the ribozyme active site than S; this indicates that the reactive phosphoryl group of S is required (McConnell et al., 1993). The second clue came from the observation that coupling is lost when the 2′-OH of G is replaced by 2′-H, indicating that the 2′-OH of G is also involved (Li & Turner, 1997). Recently, a metal ion was identified that appears to coordinate both the *pro-*S_P oxygen of the reactive phosphoryl group and the 2′-OH of G (Fig. 1, M_C; Shan et al., 2001). These results suggest that the conformational change upon substrate binding may be mediated by this bridging metal ion. Interactions with the 2′-OH of G could alter the position of M_C so that this metal ion becomes better aligned for interaction with the *pro-*S_P oxygen of the reactive phosphoryl group, and vice versa.

Thermodynamic evidence for an integral role of M_C in mediating the coupling between S and G came from mechanistic analyses of the effect of this metal ion on individual reaction steps (Shan & Herschlag, 1999). When the 2′-OH of G is replaced by 2′-NH_2 (G_N) and metal site C is occupied by Mg^{2+}, the coupling between the oligonucleotide substrate and the nucleophile is lost, presumably because of the weaker interaction of Mg^{2+} with the 2′-NH_2 than the 2′-OH group. When the Mg^{2+} ion at site C is replaced by Mn^{2+}, coupling is restored, presumably because Mn^{2+} can make a stronger interaction with the 2′-amino group than Mg^{2+}. As expected, the interaction of Mn_C^{2+} with the 2′-NH_2 of G_N requires the presence of the reactive phosphoryl group at 30 °C (Shan & Herschlag, 1999), analogous to the requirement of the reactive phosphoryl group for the coupling between S and G at this temperature (McConnell et al., 1993).

In this work, we further tested the role of M_C in the coupling between S and G by probing the interaction of M_C with the 2′-moiety of G at 4 °C; at this temperature, the guanosine nucleophile binds strongly to E even in the absence of S, most likely because low temperature favors the conformation of the ribozyme in which the substrates are already positioned to interact with M_C. Consistent with predictions from this temperature-dependent conformational change, we found that interaction of the metal ion at site C with the 2′-functional group of G can be made even in the absence of bound S at 4 °C. These results provide additional support for the proposed conformational change and for a crucial role of M_C in this change. Importantly, analysis of the data from this and the previous studies allows construction of a quantitative two-state model for this ribozyme conformational change.

RESULTS

A two-state model for a ribozyme conformational change

The coupled binding between S and G and the effect of M_C and temperature on this coupling described in the Introduction can be accounted for by a two-state model. Such a model is depicted in Figure 2 for the situation with Mn^{2+} bound at site C (Mn_C^{2+}) and with 2′-aminoguanosine (G_N) as the nucleophile. According to this model, the ribozyme exists in two conformations: (1) an "unaligned" conformation in which the reactive phosphoryl group of S and the 2′ moiety of G are not positioned to interact with M_C (top plane of Fig. 2A, B in blue), so that S and G_N bind weakly ($K_{a,rel}^{S}$ and $K_{a,rel}^{G_N}$, respectively), and (2) an "aligned" conformation in which both S and G are better positioned to interact with M_C and possibly with other active site groups (bottom plane of Fig. 2A, B in red), so that the ribozyme has higher affinities for S and G_N ($K_{a,rel}^{S}{}' > K_{a,rel}^{S}$ and $K_{a,rel}^{G_N}{}' > K_{a,rel}^{G_N}$). At 30 °C (Fig. 2A), the interactions of Mn_C^{2+} with S and G_N are required to drive the transition to the aligned conformation, that is, $K_{\Delta}^{E} < 1$ whereas $K_{\Delta}^{E\bullet S\bullet G_N} > 1$. This gives rise to the coupling between S and G and the requirement of M_C for coupling. Low temperature (Fig. 2B) favors the aligned state [i.e., there is a larger value for K_{Δ}^{E} at 4 °C (Fig. 2B) than at 30 °C (Fig. 2A)], so that S and G can bind strongly to the free ribozyme even in the absence of the other substrate. The values of the equilibrium constants in the model of Figure 2 were derived from the results described below in conjunction with the previous data, and are described in the last section of the Results.

Test of the two-state model for substrate coupling

The model in Figure 2 predicts that at low temperature, the interaction of M_C with the 2′ moiety of G will be made even in the absence of bound S. To test this model, we determined the effect of replacing the Mg^{2+} ion at site C with Mn^{2+} on the binding of G_N to the free ribozyme and to the E•S complex at 4 °C. In principle, the specific Mn_C^{2+}•G_N interaction needs to be isolated by comparing the effect of replacing Mg_C^{2+} with Mn_C^{2+} on the binding of G_N relative to G (Shan & Herschlag, 1999; Shan et al., 1999a). This analysis can be simplified, however, as previous work showed that the binding and reactivity of G and S are the same with Mg^{2+} or Mn^{2+} bound at site C (Shan & Herschlag, 1999; data not shown). Thus, this aspect of the analysis is not explicitly shown.

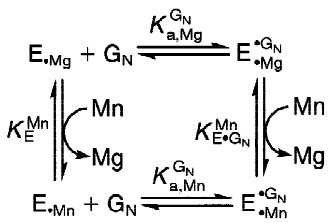
Previous work has also established conditions under which Mn^{2+} replaces Mg^{2+} at site C, showing that Mn^{2+} binds 50-fold stronger than Mg^{2+}, with apparent dissociation constants of 0.28 and 0.21 mM for free E and the E•S complex, respectively, in the presence of 10 mM Mg^{2+} (30 °C; Shan & Herschlag, 1999; Shan et al., 1999b).[3] Experiments analogous to those carried out at 30 °C showed that at 4 °C, Mn^{2+} binds to site C in free E and the E•S complex with apparent dissociation constants of 0.28 and 0.20 mM (in 10 mM Mg^{2+}), respectively, the same as the Mn_C^{2+} affinity previously observed at 30 °C (data not shown; see footnote 3).[4] The Mn_C^{2+} affinity for the E•G_N and E•S•G_N complexes are stronger than those for free E and the E•S complex; these stronger Mn_C^{2+} affinities presumably arise from the strong interaction of Mn^{2+} with the 2′-amino group of G_N in the complexes at 4 °C.[5]

To determine whether the metal ion at site C interacts with the 2′ moiety of G in the E•S•G_N complex, the binding of G_N to the E•S complex was probed in pre-steady-state kinetic experiments using the wild-type oligonucleotide substrate, rSA_5 (Table 1), with Mg^{2+} or Mn^{2+} bound at site C. To ensure that the chemical step is rate limiting, the substrate −1d,rSA_5 (Table 1) was also used; replacement of the 2′-OH of U(−1) with 2′-H slows the rate of the chemical step 10^3-fold, without

[3] Ten millimolar Mg^{2+} was present to ensure proper folding of the ribozyme and to minimize nonspecific binding of Mn^{2+} to other metal ion sites (Shan & Herschlag, 1999). Because a Mg^{2+} ion is bound at site C at Mg^{2+} concentrations above 2 mM, the experimentally determined Mn^{2+} affinities are apparent affinities, representing the exchange of Mn_C^{2+} for Mg_C^{2+} in the presence of 10 mM Mg^{2+} (Shan & Herschlag, 1999).

[4] The similar apparent Mn_C^{2+} affinities for E and the E•S complex in the presence of Mg^{2+} are consistent with the similar Mn^{2+} and Mg^{2+} affinities for oxygen ligands (Martell & Smith, 1976; Shan & Herschlag, 1999), such as the nonbridging oxygen of the reactive phosphoryl group of S, as these are ligand exchange reactions with water molecules. Thus, the presence of S would not be expected to affect the affinity of Mn^{2+} relative to Mg^{2+} at site C.

[5] These conclusions are derived from the following observations and analyses. Replacing Mg_C^{2+} with Mn_C^{2+} increases the G_N affinity for free E 20-fold at 4 °C (Table 2). Mn_C^{2+} is therefore predicted to bind to the E•G_N complex 20-fold stronger than to free E, according to the thermodynamic cycle of equation 2:



(2)

$$\frac{K_{E\bullet G_N}^{Mn}}{K_{E}^{Mn}} = \frac{K_{a,Mg}^{G_N}}{K_{a,Mn}^{G_N}} = \frac{1}{20},$$

in which K_E^{Mn} and $K_{E\bullet G_N}^{Mn}$ are the apparent Mn_C^{2+} dissociation constants from E and the E•G_N complex, respectively, and $K_{a,Mg}^{G_N}$ and $K_{a,Mn}^{G_N}$ are the G_N association constants with Mg^{2+} and Mn^{2+} bound at site C, respectively. Replacing Mg_C^{2+} with Mn_C^{2+} also increases the affinity of G_N for the E•S complex 50-fold (Table 2); an analogous thermodynamic analysis then predicts that Mn_C^{2+} binds 50-fold stronger to the E•S•G_N than the E•S complex (cf. equation 2).

affecting other reaction steps (Herschlag et al., 1993). With Mg^{2+} as the sole metal ion, G_N binds weakly to the E•S complex formed by rSA_5 and $-1d,rSA_5$, with dissociation constants of 260 and 220 μM, respectively (Fig. 3A; Table 2). In the presence of 1 mM Mn^{2+}, which is sufficient to replace Mg^{2+} at site C in the E•S complex, binding of G_N is ~40-fold stronger, with dissociation constants of 6 ± 2 μM (Fig. 3A; Table 2). As about one-sixth of the E•S complex has Mg^{2+} bound at site C at 1 mM Mn^{2+} ($K_{E\bullet S}^{Mn} = 0.20$ mM as described above),

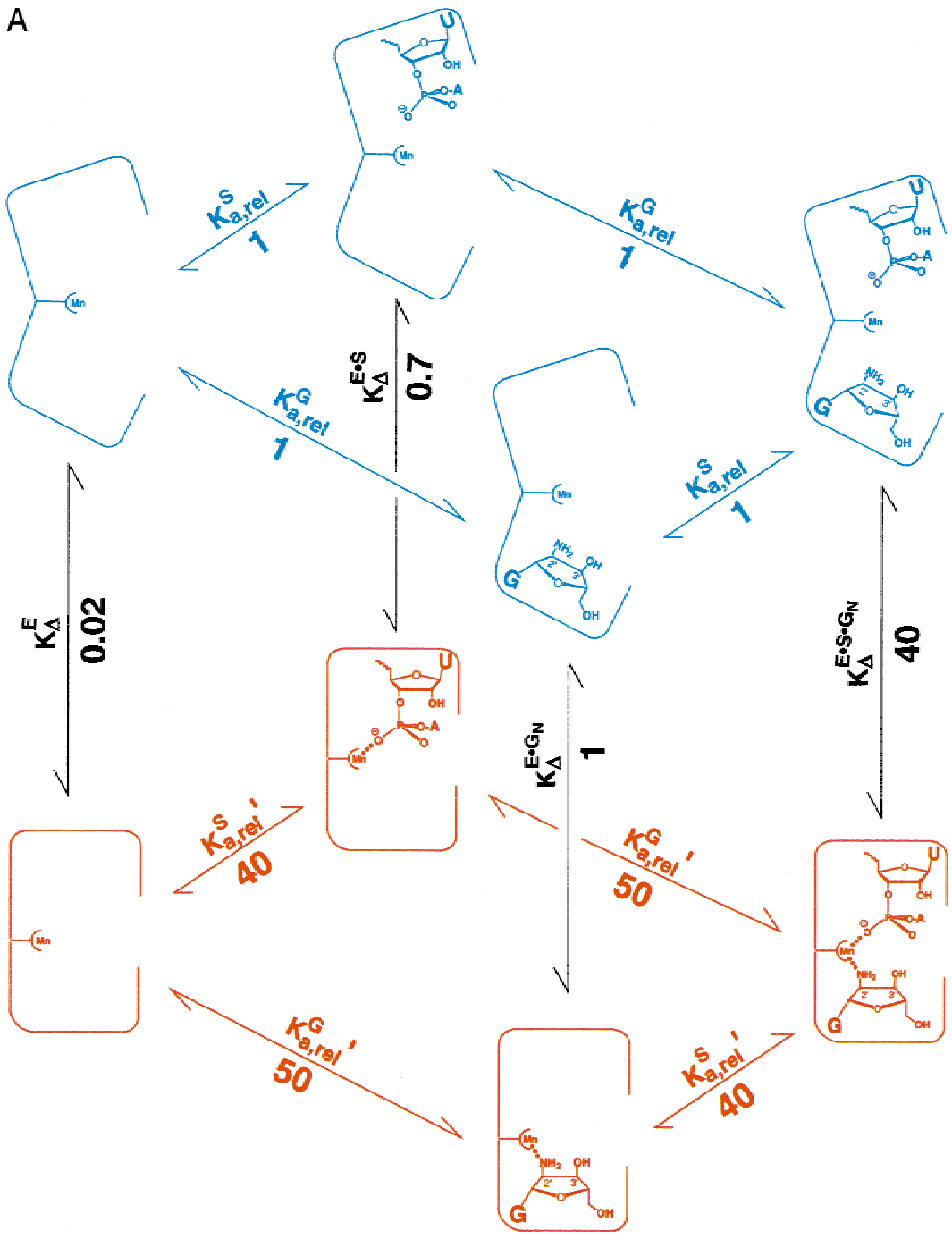


FIGURE 2. Thermodynamic framework for the conformational change of the ribozyme mediated by interactions of the metal ion at site C with the substrates at 30 °C (**A**) and 4 °C (**B**). The model is depicted for the Mn_C^{2+}•G_N pair, and was constructed using the observed Mn_C^{2+} effects on the binding of S and G_N to the ribozyme and ribozyme•substrate complexes at the two different temperatures, as described in the Results. K_a^S and $K_a^{G_N}$ denote the equilibrium association constants of S and G_N, respectively, in the unaligned state (top planes in blue), $K_a^{S\prime}$ and $K_a^{G_N\prime}$ denote the equilibrium association constants of S and G_N, respectively, in the aligned state (bottom planes in red), and K_Δ is the equilibrium constant for going from the unaligned to the aligned conformation. (*Figure continues on facing page.*)

the measured G_N dissociation constants extrapolates to a value of $K_{d,E\bullet S}^{G_N} \approx 5\ \mu M$ with Mn^{2+} fully occupying site C. This effect is analogous to previous observations at 30 °C (Table 2; Shan & Herschlag, 1999), suggesting that the Mn^{2+} at site C interacts with the 2′-amino group of G_N in the presence of bound S, that is, in the E•S•G_N complex.

To address whether Mn_C^{2+} also interacts with G_N in the absence of bound S at the active site at 4 °C, the affinity of G_N for free E was determined using the

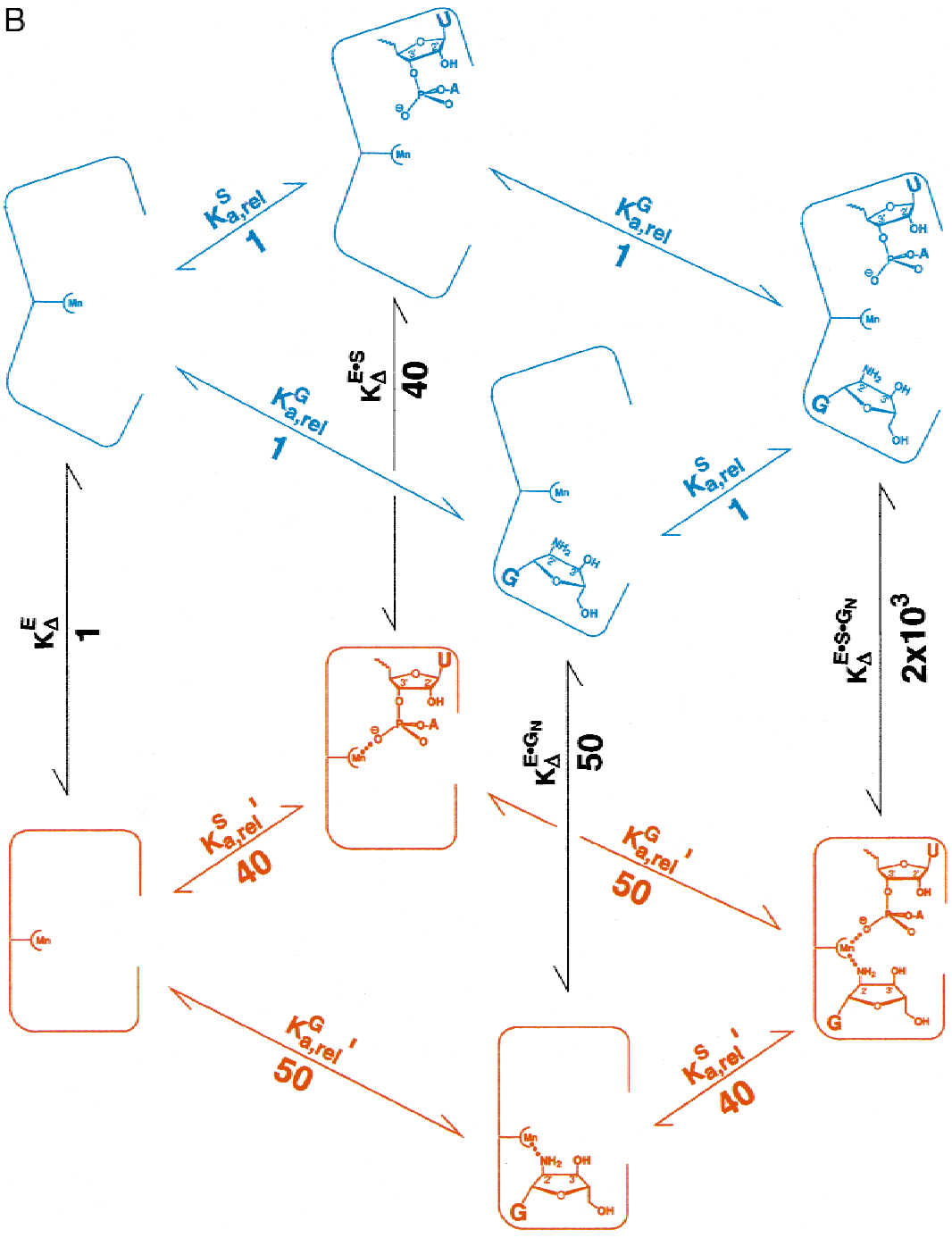


FIGURE 2. *Continued.*

TABLE 1. List of oligonucleotide substrates.[a]

Abbreviation	Oligonucleotide substrate										
	−6	−5	−4	−3	−2	−1	+1	+2	+3	+4	+5
rSA_5	rC	rC	rC	rU	rC	rU	rA	rA	rA	rA	rA
$-1d,rSA_5$	rC	rC	rC	rU	rC	dT	rA	rA	rA	rA	rA
$-3m,rSA_5$	rC	rC	rC	mU	rC	rU	rA	rA	rA	rA	rA

[a]r: 2′-OH; d: 2′-H; m: 2′-OCH_3.

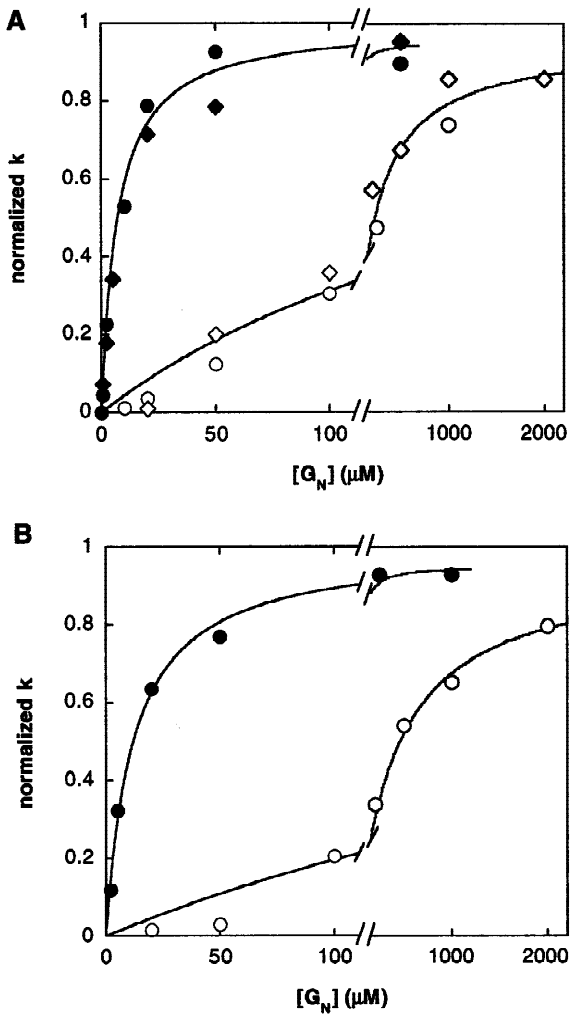


FIGURE 3. Mn^{2+} increases the affinity of G_N for free E and the E•S complex at 4 °C. **A:** G_N concentration dependence of the rate constant for reaction of rSA_5 (○, ●) and $-1d,rSA_5$ (◇,◆) in the absence or presence of 1 mM Mn^{2+} (open and closed symbols, respectively). Observed rate constants were determined at pH 7.9 (rSA_5) and 8.5 ($-1d,rSA_5$), with 100 nM E and 10 mM Mg^{2+}, as described in Materials and Methods. The observed rate constants were normalized such that $k_{norm} = 1$ with saturating G_N. The lines are fits of all of the data points at 0 or 1 mM Mn^{2+} to equation 6 in Materials and Methods, and give dissociation constants for G_N of 240 ± 20 and 6 ± 2 μM in the absence or presence of added Mn^{2+}, respectively. **B:** G_N concentration dependence of the rate constant for reaction of $-3m,rSA_5$ in the absence (○) and presence (●) of 1 mM Mn^{2+}, determined at pH 7.9 with 100 nM E and 10 mM Mg^{2+} background. The rate constants were determined and normalized as in **A**. The lines are fits of the data to equation 3, which give G_N dissociation constants of 260 ± 20 and 12 ± 2 μM in the absence and presence of added Mn^{2+}, respectively.

oligonucleotide substrate $-3m,rSA_5$ (Table 1). Previous work has shown that binding of S occurs in two steps: first, an open complex $[(E•S)_o]$ is formed, in which S binds to E solely via base-pairing interactions with the internal guide sequence of E to form a P1 duplex without active site interactions; second, a closed complex $[(E•S)_c]$ is formed, in which the P1 duplex docks into the ribozyme active site via tertiary interactions (Bevilacqua et al., 1992; Herschlag, 1992; Narlikar & Herschlag, 1996; Narlikar et al., 1997). The wild-type oligonucleotide substrate rSA_5 binds E predominantly in the closed complex, whereas modified substrates such as $-3m,rSA_5$ binds E predominantly in the open complex (Narlikar & Herschlag, 1996; Narlikar et al., 1997). Several previous results have shown that the $(E•S)_o$ complex formed by $-3m,rSA_5$ provides a reasonable and convenient model for probing the interactions of guanosine or active site metal ions with the free ribozyme, because S does not interact with active site groups in the open complex (Knitt et al., 1994; Shan et al., 1999a; Shan & Herschlag, 1999). Direct determination of the docking of $-3m,rSA_5$ indicates that this substrate remains predominantly undocked at temperatures down to 10 °C, with the trend toward weaker docking as temperature is low-

TABLE 2. Equilibrium dissociation constants for binding of G_N to E and the E•S complex with Mg^{2+} or Mn^{2+} bound at site C at 4 and 30 °C.

	$K_d^{G_N}$ (μM)			
	E		E•S	
Temp (°C)	Mg_C^{2+}	Mn_C^{2+}	Mg_C^{2+}	Mn_C^{2+}
30	240[a]	110[a]	280[a]	10[a]
4	260[b]	10[b]	220,[c] 260[d]	5[e]

[a]Values are from Shan and Herschlag (1999).

[b]The G_N affinity for free E was assumed to be equal to that for the $(E•S)_o$ complex, as described in the text, and was determined with $-3m,rSA_5$ (Table 1). The observed G_N affinity at 1 mM Mn^{2+} was extrapolated to the value with Mn^{2+} fully occupying site C, as described in the text.

[c]Determined with $-1d,rSA_5$ (Table 1).

[d]Determined with rSA_5 (Table 1).

[e]Determined with rSA_5 and $-1d,rSA_5$ in the presence of 1 mM Mn^{2+} and extrapolated to the value with Mn^{2+} fully occupying site C, as described in the text.

ered (L. Bartley, X. Zhang, R. Das, S. Chu, & D. Herschlag, unpubl. results).

In the absence of Mn^{2+}, G_N binds to the $(E•S)_o$ complex with a dissociation constant of 260 μM (Fig. 3B; Table 2), analogous to the weak G_N affinity previously observed at 30 °C with Mg^{2+} as the sole metal ion (Shan & Herschlag, 1999). However, in the presence of 1 mM Mn^{2+}, sufficient to occupy site C, the affinity of G_N for free E is 20-fold stronger, with a dissociation constant of 12 μM (Fig. 3B; Table 2). As about one-fifth of the free ribozyme has Mg^{2+} bound at site C in the presence of 1 mM Mn^{2+} ($K_E^{Mn} = 0.28$ mM, as described above), the observed G_N affinity extrapolates to a value of $K_{d,E}^{G_N} \approx 10$ μM with Mn^{2+} fully occupying site C. The ability of Mn_C^{2+} to increase the G_N affinity of free E is in contrast to previous observations at 30 °C, at which Mn_C^{2+} has only a twofold effect on the binding of G_N to free E and the $(E•S)_o$ complex (Table 2; Shan & Herschlag, 1999). Thus, at 4 °C, the metal ion at site C can interact strongly with the 2′ moiety of G even in the absence of S bound at the active site.

Construction of the quantitative two-state model for the ribozyme conformational change

As described above in the first section of the Results, a two-state model with an unaligned and an aligned conformation can account for the previous observations (Fig. 2). The results from this work, combined with data previously obtained, allow us to quantitatively estimate the individual equilibrium constants in Figure 2. The available experimental data allow only a very crude estimate of the individual equilibrium constants, so only one significant number is reported in Figure 2.

The following assumptions were made in obtaining the individual equilibrium constants of this framework. First, in defining a two-state model, it is assumed that S and G do not affect the binding affinity of one another within each conformational state; rather, the effect of S and G binding is to shift the conformational equilibrium of the ribozyme towards the aligned state. Thus in each conformational state, the affinity of S for free E is the same as its affinity for the E•G_N complex, and likewise the G_N affinity for free E is the same as its affinity for the E•S complex. For simplicity of illustration, the S affinity is represented by a single association constant in each conformational state, $K_{a,rel}^{S}$ in the unaligned state (top plane in blue) and $K_{a,rel}^{S}{}'$ in the aligned state (bottom plane in red). Analogously, the G_N affinity is represented by $K_{a,rel}^{G_N}$ in the unaligned state and $K_{a,rel}^{G_N}{}'$ in the aligned state. We also assume that the magnitude of increase in substrate affinity in going from the unaligned to the aligned state is the same regardless of temperature.

These assumptions are supported by the following observations. First, there is little variation in the affinity of G for the E•S complex over a wide temperature range (4–50 °C; McConnell & Cech, 1995), suggesting that the G affinity for the aligned state is independent of temperature. Second, the binding of G to free E is largely the same from 30 to 50 °C, the temperature range within which the free ribozyme is predominantly in the unaligned state. These observations suggest that the guanosine substrate affinity for the unaligned state is also independent of temperature. Consideration of these observations and the thermodynamic cycles in Figure 2 supports the assumption that the magnitude of increase in G (or G_N) affinity in going from the unaligned to the aligned state is independent of temperature. Finally, the affinity of G for free E at 4 °C is essentially the same as its affinity for E•S at all temperatures (McConnell & Cech, 1995), and, likewise, the affinity of G_N for free E at 4 °C is essentially the same as its affinity for E•S at both 0 and 30 °C in the presence of Mn_C^{2+} (Shan & Herschlag, 1999; Table 2), suggesting that bound S does not affect the binding of G or G_N in the aligned state. These observations support the two-state assumption that S and G do not affect the affinity of one another in the aligned state. There is no direct experimental evidence for the absence of an effect of S and G on the binding of one another in the unaligned state. Nevertheless, as defined in the minimal two-state model, there is no direct or indirect interactions between S and G in the unaligned state. A breakdown of the assumption that the increase in substrate binding is temperature independent would alter the equilibrium constants in this quantitative two-state model, but most likely would not cause a breakdown of the two-state model. On the other hand, if the two-state assumption did not hold, more complicated models involving additional conformational states would need to be included.

Estimation of substrate affinities in the aligned and unaligned conformation (Fig. 2, top and bottom planes)

For simplicity of illustration, the affinity of the ribozyme for S and G_N are described *relative* to the affinities in the unaligned conformation, so that the *relative* substrate affinities in the unaligned conformation are defined as equal to 1 ($K_{a,rel}^{S} \equiv 1$ and $K_{a,rel}^{G_N} \equiv 1$).

With Mg^{2+} bound at site C, G_N binds with a weak affinity to free E at high temperature (30 °C, $K_d^{G_N} =$ 250 μM; Shan & Herschlag, 1999); this affinity can therefore be used as a good approximation for the G_N affinity in the unaligned conformation, in which the 2′ moiety of G does not interact with M_C. In the presence of Mn_C^{2+}, binding of G_N to the E•S complex is 20-fold stronger than with Mg^{2+} bound at site C, with a dissociation constant of 12 μM. Lowering the temperature to 4 °C has only an additional twofold effect on this G_N affinity, giving a dissociation constant of 5 μM from the E•S•G_N complex (Table 2),

despite the fact that lowering temperature from 30 to 4 °C substantially shifts the conformational equilibrium of the ribozyme towards the aligned state (see below). This suggests that as the conformational equilibrium is shifted towards the aligned state, the G_N affinity plateaus at a value of ~5 μM. This then represents the G_N affinity in the aligned conformation, which is 50-fold higher than the affinity in the unaligned conformation ($K_{a,rel}^{G_N\prime} = 250/5 \approx 50$).

At 30 °C, replacing the Mg^{2+} at site C with Mn^{2+} increases the affinity of S for the E•G_N complex by 18-fold (Shan & Herschlag, 1999), presumably because the stronger interaction of Mn^{2+} than Mg^{2+} with G_N shifts the conformational equilibrium of the E•G_N complex towards the aligned state, in which S binds stronger than in the unaligned state. The G_N dissociation constant for the E•G_N complex in the presence of Mn_C^{2+} suggests that half of the E•G_N complex is in the aligned conformation at 30 °C (see next section). The magnitude of the strengthening of S binding in going from the unaligned to the aligned conformation can then be shown to be twice the observed amount, giving $K_{a,rel}^{S\prime} \approx 40$.[6]

Estimate of the conformational equilibrium at 30°C (Fig. 2A, K_Δ)

The value of the conformational equilibrium constant for the E•G_N complex can be quantitatively derived from the following analysis. In the presence of bound Mn_C^{2+} the observed G_N affinity is twofold higher than the G_N affinity with Mg_C^{2+} bound; the affinity with bound Mg_C^{2+} represents the G_N affinity in the unaligned state (Shan & Herschlag, 1999). As the observed G_N affinity is a weighted average of the G_N affinities of the ribozyme in the aligned and the unaligned state, this twofold higher affinity indicates that half of the E•G_N complex is in the aligned state.[7] Thus, the conformational equilibrium in the E•G_N complex is close to 1 ($K_\Delta^{E\bullet G_N} \approx 1$).

The conformational equilibrium for the E•S complex can be obtained from an analogous analysis. In the presence of Mn_C^{2+}, the affinity of the E•S complex for G_N at 30 °C is twofold lower than that observed at 4 °C, which is taken to represent the G_N affinity in the aligned conformation (see previous section). This twofold lower G_N affinity then suggests that about half of the E•S complex is in the aligned state at 30 °C.[8] Thus, the conformational equilibrium in the E•S complex is also close to 1 ($K_\Delta^{E\bullet S} = 0.7$).

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[6] The observed S affinity is the weighted average of the S affinity for the E•G_N complex in the aligned and the unaligned state. This is described quantitatively by

$$K_{a,obsd}^{S} = F \times K_a^S + (1 - F) \times K_a^{S\prime}, \tag{3a}$$

which can be rearranged to give

$$K_a^{S\prime} = \frac{K_{a,obsd}^{S} - F \times K_a^S}{1 - F}, \tag{3b}$$

where K_a^S and $K_a^{S\prime}$ are the S affinities in the unaligned and aligned states, respectively, F is the fraction of E•G_N in the unaligned state, and $K_{a,obsd}^S$ is the observed S affinity. There is evidence that half of the E•G_N complex is in the aligned state, that is, $F = 0.5$ (see text). The 18-fold increase in S binding when Mn_C^{2+} replaces Mg^{2+} suggests that the observed S affinity is 18-fold larger than the S affinity in the unaligned state, that is, $K_{a,obsd}^S = 18\ K_a^S$ (see text). Substituting these values into equation 3b gives $K_a^{S\prime} \approx 40\ K_a^S$.

[7] The observed G_N dissociation constant is the weighted average of the G_N dissociation constants for the E•G_N complex in the aligned and the unaligned states. This is quantitatively described by

$$K_{d,obsd}^{G_N} = F \times K_d^{G_N} + (1 - F) \times K_d^{G_N\prime}, \tag{4a}$$

which can be rearranged to

$$F = \frac{K_{d,obsd}^{G_N} - K_d^{G_N\prime}}{K_d^{G_N} - K_d^{G_N\prime}}, \tag{4b}$$

where F is the fraction of the E•G_N complex in the unaligned state, $K_d^{G_N}$ and $K_d^{G_N\prime}$ are the G_N dissociation constants in the unaligned and aligned states, respectively ($K_d^{G_N} = 1/K_a^{G_N}$), and $K_{d,obsd}^{G_N}$ is the observed G_N dissociation constant. As noted in the text, results from this and previous work suggest the following: $K_d^{G_N} = 250\ \mu$M, $K_d^{G_N\prime} = 5\ \mu$M, and $K_{d,obsd}^{G_N} = 110\ \mu$M (Shan & Herschlag, 1999). Substituting these values into equation 4 gives $F = 0.43$. The conformational equilibrium for the E•G_N complex can then be calculated as: $K_\Delta^{E\bullet G_N} = (1 - F)/F = 1.3 \approx 1$.

[8] Analogous to the analysis in footnote 7, the observed G_N affinity is the weighted average of G_N affinities for the E•S complex in the aligned and the unaligned states. This is described by

$$K_{a,obsd}^{G_N} = F \times K_a^{G_N} + (1 - F) \times K_a^{G_N\prime}, \tag{5a}$$

which can be rearranged to give

$$F = \frac{K_a^{G_N\prime} - K_{a,obsd}^{G_N}}{K_a^{G_N\prime} - K_a^{G_N}}, \tag{5b}$$

where F is the fraction of E•S complex in the unaligned state, $K_a^{G_N}$ and $K_a^{G_N\prime}$ are the G_N association constants in the aligned and unaligned states, respectively, and $K_{a,obsd}^{G_N}$ is the observed G_N association constant. As noted in the text, the results from this and previous work suggest the following:

$$K_a^{G_N} \equiv \frac{1}{K_d^{G_N}} = \frac{1}{250\ \mu\text{M}} = 4\ \text{mM}^{-1},$$

$$K_a^{G_N\prime} \equiv \frac{1}{K_d^{G_N\prime}} = \frac{1}{5\ \mu\text{M}} = 200\ \text{mM}^{-1},$$

and $K_{a,obsd}^{G_N} = 83\ \text{mM}^{-1}$ (Shan & Herschlag, 1999). Substituting these values into equation 5 gives $F = 0.6$. The conformational equilibrium of the E•S complex can then be calculated: $K_\Delta^{E\bullet S} = (1 - F)/F = 0.7$. An analogous analysis can be made to obtain the

The conformational equilibrium for free E can then be estimated from these equilibrium constants and the 40-fold stronger S binding (or 50-fold stronger G_N binding) in the aligned than the unaligned conformation, according to the following relationship derived from the thermodynamic cycle in Figure 2A:

$$K_\Delta^E = K_\Delta^{E\bullet S} \times \frac{K_{a,rel}^S}{K_{a,rel}^{S\prime}} = 0.7 \times \frac{1}{40} \approx 0.02$$

$$\left(\text{or } K_\Delta^E = K_\Delta^{E\bullet G_N} \times \frac{K_{a,rel}^{G_N}}{K_{a,rel}^{G_N\prime}} = 1 \times \frac{1}{50} = 0.02\right).$$

The conformational equilibrium for the E•S•G_N complex can be analogously estimated from the conformational equilibrium for E•S (or E•G_N) and the 40-fold stronger G_N (or S) binding in the aligned conformation, according to the thermodynamic relationship:

$$K_\Delta^{E\bullet S\bullet G_N} = K_\Delta^{E\bullet S} \times \frac{K_{a,rel}^{G_N\prime}}{K_{a,rel}^{G_N}} = 0.7 \times 50 = 40$$

$$\left(\text{or } K_\Delta^{E\bullet S\bullet G_N} = K_\Delta^{E\bullet G_N} \times \frac{K_{a,rel}^{S\prime}}{K_{a,rel}^{S}} = 1 \times 40 = 40\right).$$

Estimate of the conformational equilibrium at 4 °C (Fig. 2B, K_Δ)

At 4 °C, Mn_C^{2+} increases the affinity of the free ribozyme for G_N by 20-fold, in contrast to the absence of Mn_C^{2+} on the binding of G_N to free E at 30 °C; this suggests that a larger fraction of free E is in the aligned conformation at 4 °C than at 30 °C. However, the G_N affinity for free E is still twofold smaller than that for the E•S complex at this temperature (Table 2), which is taken to represent the G_N affinity in the aligned conformation (see the section, Estimation of substrate affinities in the aligned and unaligned conformation, above). As the observed G_N affinity is the weighted average of the affinities in the aligned and the unaligned state, a conformational equilibrium of 1 can be calculated for the free ribozyme at 4 °C ($K_\Delta^E = 1$). (See footnote 8.)

The conformational equilibrium for the E•S complex can then be estimated from this equilibrium value and the 40-fold stronger S binding in the aligned than the unaligned conformation, according to the following relationship derived from the thermodynamic cycle in Figure 2B:

$$K_\Delta^{E\bullet S} = K_\Delta^E \times \frac{K_{a,rel}^{S\prime}}{K_{a,rel}^{S}} = 1 \times 40 = 40.$$

Analogously, the conformational equilibrium for the E•G_N complex can be estimated from the relationship:

$$K_\Delta^{E\bullet G_N} = K_\Delta^E \times \frac{K_{a,rel}^{G_N\prime}}{K_{a,rel}^{G_N}} = 1 \times 50 = 50.$$

These values then allow the conformational equilibrium for the E•S•G_N to be estimated:

$$K_\Delta^{E\bullet S\bullet G_N} = K_\Delta^{E\bullet S} \times \frac{K_{a,rel}^{G_N\prime}}{K_{a,rel}^{G_N}} = 40 \times 50 = 2 \times 10^3$$

$$\left(\text{or } K_\Delta^{E\bullet S\bullet G_N} = K_\Delta^{E\bullet G_N} \times \frac{K_{a,rel}^{S\prime}}{K_{a,rel}^{S}} = 50 \times 40 = 2 \times 10^3\right).$$

DISCUSSION

Role of conformational change in the function of RNA molecules

Previous work provided strong evidence for a conformational change upon substrate binding in the *Tetrahymena* ribozyme and led to a model in which the metal ion at site C plays a central role in mediating this conformational change. This study has tested a direct prediction of this model and provided additional evidence for its validity. The question thus arises: What is the role of the aligned conformation in catalysis of the *Tetrahymena* ribozyme? It is important to recognize that the conformational change per se investigated in this work does not have an apparent role in catalysis. Rather, the aligned conformation is presumably a functional state or, at least, closer to the catalytic conformation of this ribozyme. Replacing the 2′-OH of G with 2′-H reduces the rate of the reaction $\geq 10^6$-fold (Bass & Cech, 1986; Tanner & Cech, 1987; data not shown), and replacing the *pro-*S_P oxygen of the reactive phosphoryl group with a sulfur atom reduces the reaction rate 10^3-fold (Rajagopal et al., 1989; Yoshida et al., 2000; Shan et al., 2001), indicating that the ligands of M_C are very important for catalysis by this RNA enzyme. Considering the geometrical requirements for attack of the guanosine nucleophile at the reactive phosphorus, it is likely

conformational equilibrium for free E at 4 °C. In this case, the observed G_N affinity is

$$K_{a,obsd}^{G_N} = \frac{1}{K_{d,obsd}^{G_N}} = \frac{1}{10\ \mu M} = 100\ mM^{-1}$$

(Table 2). Substituting this observed affinity and the above values for $K_a^{G_N}$ and $K_a^{G_N\prime}$ into equation 5 gives $F = 0.5$ and $K_\Delta^E = (1 - F)/F = 1$.

In this and footnote 7, the G_N association constant, $K_a^{G_N}$, and the dissociation constant, $K_d^{G_N}$, were used for the analysis depending on whether the E•S or E•G_N complex is the focus of discussion. $K_a^{G_N}$ monitors the association process: E•S + $G_N \rightarrow$ E•S•G_N, such that analysis of $K_a^{G_N}$ directly gives the conformational equilibrium for the E•S complex. On the other hand, $K_d^{G_N}$ monitors the dissociation process: E•$G_N \rightarrow$ E + G_N, such that analysis of $K_d^{G_N}$ directly gives the conformational equilibrium for the E•G_N complex (Fersht, 1984).

that in the aligned conformation, the bridging interactions of M_C with S and G help position the guanosine nucleophile and the reactive phosphoryl group with respect to one another and possibly with respect to other catalytic groups at the active site, thereby facilitating the reaction (Jencks, 1975). The interaction of M_C with the nonbridging reactive phosphoryl oxygen may also help to stabilize negative charge development on the nonbridging oxygen in the transition state, thereby contributing to catalysis.

In general, conformational changes are required if the resting form of the RNA molecule is not the functionally active form. For example, some small RNA molecules do not fold into their active structure even in the presence of substrates, so that conformational changes are often considered important for their function (Patel et al., 1997; Frankel & Smith, 1998; Wang et al., 1999; Doherty & Doudna, 2001). On the other hand, larger RNA molecules such as the *Tetrahymena* ribozyme and some smaller ribozymes have sometimes been thought to possess a largely preorganized structure (Cate et al., 1996; Ferre-D'Amare et al., 1998; Golden et al., 1998; Herschlag, 1998; Doherty & Doudna, 2001). Nevertheless, although the global structure of some RNA molecules may be closer to their active conformations, local rearrangements of the active site may still be required.

Although conformational changes can be integral to function (see below), conformational changes should not be assumed a priori to occur because of a functional imperative. Conformational changes may arise from the limitations in specifying a unique structure from the limited building blocks available to RNA (Narlikar & Herschlag, 1997). The *Tetrahymena* RNA undergoes additional conformational changes besides the conformational change investigated in this work. The E•S complex changes from an open to a closed complex upon substrate binding, as described above (Pyle & Cech, 1991; Bevilacqua et al., 1992; Herschlag, 1992; Strobel & Cech, 1993; Knitt et al., 1994; Narlikar & Herschlag, 1996; Narlikar et al., 1997; Strobel et al., 1998). An additional conformational change upon G binding has also been suggested (Herschlag & Khosla, 1994), and appears to be distinct from the conformational change that is the focus of this work (K. Karbstein & D. Herschlag, unpubl. results). Additional conformational changes involving metal ions A and B and their ligands are also possible, as discussed in the next section.

Is there a biological role for the *Tetrahymena* ribozyme conformational changes? Considering the self-splicing reaction of group I introns, one or more conformational changes are needed between the first and second steps of splicing (Golden & Cech, 1996). The conformational change may be involved in the array of structural rearrangements that are necessary between the two steps in the splicing of group I introns. Multiple conformational changes have also been extensively described for mRNA splicing, for the self-splicing of group II introns, and for ribosomal RNAs (Costa et al., 1997; Lodmell & Dahlberg, 1997; Staley & Guthrie, 1998; Wilson & Noller, 1998). In general, conformational changes will be crucial for the function of RNA molecules that carry out complex, multistep processes and/or processes that require control and regulation.

Quantitative analysis as a tool for probing RNA conformational changes

The thermodynamic model described in this work provides a conceptual and quantitative framework that will allow us and others to probe additional molecular interactions involved in this conformational change, to understand its functional role, and to identify and distinguish additional conformational changes in the *Tetrahymena* ribozyme. For example, the metal ion that coordinates the nucleophilic 3′-oxygen of G (Fig. 1, M_B) binds weakly to the ribozyme, and its affinity is unaffected by G binding, raising the possibility that metal site B is not positioned to coordinate the 3′-OH of G in the E•G complex (30 °C; Shan et al., 1999a). Is M_B better positioned in the aligned conformation, or is positioning of M_B independent of M_C and its interactions? This can be tested by measuring the interaction of M_B with the 3′ moiety of G at 4 °C, as the ribozyme is then predominantly in the aligned conformation. Another active site metal ion, M_A, coordinates both the 3′ bridging oxygen of S and the *pro-*S_P oxygen of the reactive phosphoryl group (Fig. 1). Is a conformational change in the E•S complex required to make the M_A interactions? If so, is it the same conformational change as that described in this work? The conditions established herein for obtaining ribozymes in predominantly the aligned or unaligned conformation will facilitate experiments that address these questions. In general, functional studies combined with thermodynamic analysis analogous to the work described herein can provide a powerful tool for the investigation of RNA conformational rearrangements.

The quantitative model described in this work also sheds new light on the mechanism of molecular recognition by RNA molecules. Complex formation between RNA and their ligands, proteins, or other RNA molecules have often been discussed in terms of mechanisms such as "induced-fit" or "prealigned" (e.g., Cate et al., 1996; Patel et al., 1997; Frankel & Smith, 1998; Golden et al., 1998). According to this categorization, however, the mechanism for substrate recognition by the *Tetrahymena* RNA seems to change under different conditions. As described herein, at 30 °C the ribozyme•substrate interactions fall into the category of the induced-fit mechanism, in which the interactions of M_C with S or G are required to provide the energy to drive a conformational change of the ribozyme to a more aligned conformation. In contrast, upon shifting to

lower temperature (4 °C) the ribozyme structure becomes prealigned, so that the substrate interactions are no longer required to drive this conformational change. These results demonstrate that quantitative differences in the conformational equilibrium of a biological molecule under different conditions can lead to qualitatively different descriptions of its interaction mechanisms. Extensive quantitative analysis will be required to develop a deep and thorough understanding of binding interactions, RNA conformational states, and the transitions between these states.

MATERIALS AND METHODS

Materials

L-21 *Sca*I ribozyme was in vitro transcribed and purified as described (Zaug et al., 1988). Oligonucleotides were gifts from L. Beigelman and were prepared and 5′-end labeled using standard methods (Zaug et al., 1988). 2′-Aminoguanosine was a gift from Dr. F. Eckstein.

General kinetic methods

All reactions were single turnover, with E in excess of labeled S (S*) and were carried out in 50 mM buffer in a background of 10 mM $MgCl_2$. Ribozyme was folded for 30 min at 50 °C, and then slowly cooled to 4 °C before initiation of the reaction, as described previously (Herschlag & Cech, 1990; McConnell et al., 1993). Reactions were followed and analyzed as previously described (Herschlag & Cech, 1990; Shan & Herschlag, 1999).

Determination of G_N affinities

The equilibrium dissociation constants of G_N, $K_d^{G_N}$, were determined from fits of the G_N concentration dependencies of the reaction to the following equation as described (Shan & Herschlag, 1999):

$$k_{obsd} = k_{max} \times \frac{[G_N]}{[G_N] + K_d^{G_N}} \quad , \qquad (6)$$

where k_{obsd} is the observed rate constant for the reaction at a given G_N concentration, and k_{max} is the maximal rate constant observed with saturating G_N. Previous work has shown that the 2′-amino group of G_N is predominantly deprotonated under the conditions used in this work (Aurup et al., 1994; Shan et al., 1999b).

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