Use of Duplex Rigidity for Stability and Specificity in RNA Tertiary Structure†

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ABSTRACT: The *Tetrahymena* group I ribozyme's oligonucleotide substrate, $CCCUCUA_5$, forms six base pairs with the ribozyme's internal guide sequence (IGS, 5′GGAGGG) to give the P1 duplex, and this duplex then docks into the active site via tertiary interactions. Shortening the substrate by three residues to give $UCUA_5$ reduces the equilibrium constant for P1 docking by ∼200-fold even though $UCUA_5$ retains all the functional groups known to be involved in tertiary interactions [Narlikar, G. J., Bartley, L. E., Khosla, M., and Herschlag, D. (1999) *Biochemistry 38*, 14192−14204]. Here we show that the P1 duplex formed with $UCUA_5$ engages in all of the major tertiary interactions made by the standard P1 duplex. This suggests that the destabilization is not due to disruption of specific tertiary interactions. It therefore appears that the weaker docking of $UCUA_5$ arises from the increased conformational freedom of the undocked P1 duplex, which has three unpaired IGS residues and thus a larger entropic cost for docking. Further, a 2′-methoxy substitution at an IGS residue that is base-paired in the standard P1 duplex with $CCCUCUA_5$ but unpaired in the P1 duplex with $UCUA_5$ destabilizes docking of the standard P1 duplex ∼300-fold more than it destabilizes docking of the P1 duplex formed with $UCUA_5$. These results suggest that fixation of groups in the context of a rigid duplex may be a general strategy used by RNA to substantially increase interaction specificity, both by aiding binding of the desired functional groups and by increasing the energetic cost of forming alternative interactions.

The low diversity of RNA side chains and the high charge and conformational flexibility of the RNA backbone might be expected to limit the close packing and precise positioning of functional groups required to form stable and well-defined RNA structures (*1*, *2*). Nevertheless, it is evident from the recent crystal structures of the *Tetrahymena* group I and hepatitis delta virus ribozymes that RNA can use various strategies to overcome these limitations (*3*−*6*). The extensive helix−helix interactions in these structures suggest that one way by which RNA can achieve precise positioning of groups interacting in tertiary structure is by presenting them in the context of secondary structural elements. The greater rigidity of a duplex compared to a single strand is expected to reduce the entropic cost of making specific interactions.

The well-characterized binding reaction of the *Tetrahymena* ribozyme's oligonucleotide substrate provides an opportunity to test and quantitate the role of duplex rigidity in facilitating RNA/RNA interactions. Binding of the oligonucleotide substrate $CCCUCUA_5$ (S)[1] entails P1 duplex formation with the ribozyme's internal guide sequence (IGS) to give an open complex, followed by docking of the P1 duplex into the catalytic core via specific tertiary interactions to give a closed complex (Figure 1) (*7*−*10*). We previously observed that removal of three residues from the substrate to give $UCUA_5$ destabilizes the closed complex by ∼200-fold [3.4 kcal/mol, 50 °C (*11*)]. Here, we have investigated the origin of this destabilization. The results suggest that the substantial destabilization arises because a greater loss in entropy is required for positioning the P1 duplex formed with $UCUA_5$. The results further suggest that conformational constraints imposed by duplex formation greatly increase the specificity of tertiary interactions made by P1 functional groups.

MATERIALS AND METHODS

Materials. L-21 ScaI ribozyme was prepared by in vitro transcription and purified as described previously (*12*). The dG22, I22, and dG25 ribozymes were prepared by ligation

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[1] Abbreviations: E refers generically to the *Tetrahymena* group I L-21 *Sca*I ribozyme and its modified versions. IGS refers to the internal guide sequence of the ribozyme, 5′GGAGGG (see Figure 1A), and G refers to the guanosine cofactor. The individual ribozymes are defined with respect to modifications in their IGS sequences. Thus, WT refers to the standard *Tetrahymena* ribozyme, dG22 and dG25 refer to ribozymes with 2′-deoxyribose substitutions at positions 22 and 25 of the IGS, respectively, mG25 refers to a ribozyme with a 2′-deoxy 2′-methoxy substitution at position 25, and I22 refers to a ribozyme having the guanosine at position 22 replaced with an inosine. S refers to the oligonucleotide substrate, without specification of length, and S* to [^{32}P]-5′-end labeled S. mU refers to a 2′-deoxy-2′-methoxy substitution and dU to a 2′-deoxy substitution at a U residue. Thus, $CCCmUCUA_5$ refers to a substrate with a 2′-methoxy-2′-deoxyribose residue at position −3 (positions defined in Figure 1B) and ribose residues at all other positions. The superscript (P1) refers to the P1 duplex formed between WT ribozyme and either the full-length substrate or $UCUA_5$, and the superscript (modified P1) refers to the corresponding P1 duplex having a 2′-deoxy, 2′-deoxy-2′-methoxy, or G → I substitution. NaMES, is sodium 2-(*N*-morpholino)ethanesulfonate; EDTA, (ethylenedinitrilo)-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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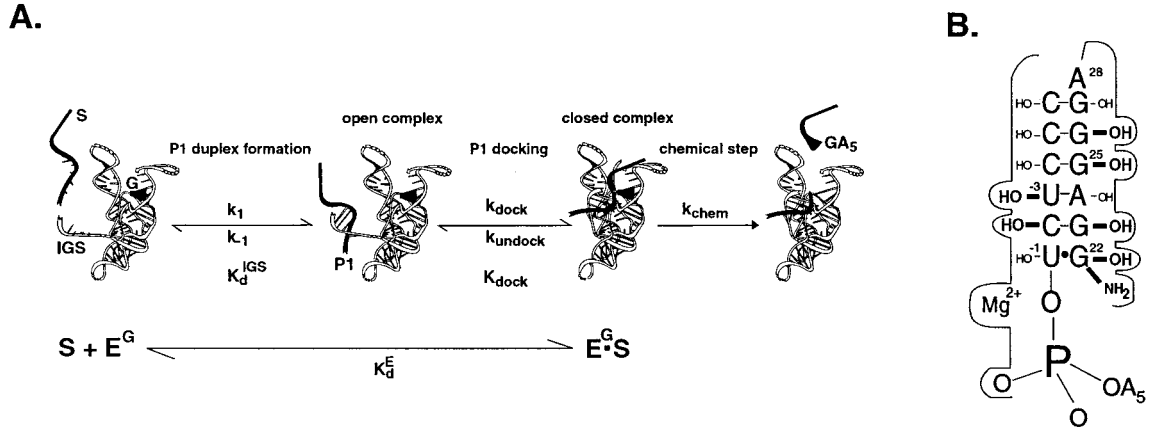

FIGURE 1: Binding of the oligonucleotide substrate. (A) The oligonucleotide substrate binds in two steps prior to catalytic cleavage (*7*−*9*, *31*). The substrate (S) first base-pairs with the internal guide sequence (IGS) of the ribozyme to form the P1 duplex in the open complex. The P1 duplex then docks into the catalytic core via specific tertiary interactions to give a closed complex. The docked substrate is cleaved by a bound exogenous guanosine molecule (G). K_d^E is the observed equilibrium constant for dissociation of S from the E^G•S complex, K_d^{IGS} is the equilibrium constant for dissociation of S from the open complex, and K_{dock} is the equilibrium constant for docking into the tertiary interactions. (B) The oligonucleotide substrate is held by both base pairing interactions with the internal guide sequence of the ribozyme (IGS ≡ 5′GGAGGG) and tertiary interactions (*14*, *15*, *17*, *22*, *32*−*37*). The ribozyme active site is represented schematically by the outline. The larger 2′-OH groups and the exocyclic amino group of the G•U wobble pair shown in boldface type provide tertiary stabilization to the P1 duplex. Panel (A) is adapted from an original drawing by L. Jaeger.

of a synthetic 17 base oligonucleotide to a shortened RNA transcript (L-38 ScaI) with T4 DNA ligase and a DNA splint as described previously (*13*, *14*). The mG25 ribozyme was a gift from Scott Strobel. Oligonucleotides were made by solid-phase synthesis and were supplied by ClonTec (Palo Alto, CA), Oligos Etc., or the Protein and Nucleic Acid Facility at Stanford or were used and characterized in previous studies. Oligonucleotide substrates were 5′-end-labeled with approximately equimolar amounts of [γ-^{32}P]-ATP by T4 polynucleotide kinase and purified by nondenaturing polyacrylamide gel electrophoresis, as described previously (*12*, *15*).

General Kinetic Methods. All reactions were single-turnover, with ribozyme (E, 2−12 000 nM) in excess of 5′-end-labeled oligonucleotide substrate (S*, ∼0.01−0.05 nM), and were carried out at 50 °C in 50 mM NaMES buffer, pH 6.6, and 10 mM $MgCl_2$ (*15*, *16*) with either 0.02 or 2 mM guanosine (G). Reactions were initiated by addition of S* following a 15 min/50 °C preincubation of E in $MgCl_2$ and buffer. Six aliquots of 1−2 μL were removed from 20 μL reactions at specified times, and further reaction was quenched by the addition of ∼2 volumes of 20 mM EDTA in 90% formamide with 0.005% xylene cyanol, 0.01% bromophenol blue, and 1 mM Tris, pH 7.5. Substrate and product(s) were separated by electrophoresis on 20% polyacrylamide/7 M urea gels, and their ratio at each time point was quantitated with a Molecular Dynamics PhosphorImager. Fast reactions were followed for ∼3$t_{1/2}$. The slow reactions of dG25, dG22, and I22 with $UCUA_5$ were followed up to ∼40% reaction, reactions of mG25 with $UCUA_5$ up to 4% reaction, and reactions of WT ribozyme with $\underline{dU}CUA_5$ and $\underline{mU}CUA_5$ up to 10% and 5% reaction, respectively, because ribozyme activity decreased at longer times. End points of 95% were obtained and used in nonlinear first-order fits to the data (KaleidaGraph, Synergy Software, Reading, PA). Reactions for which only initial rates could be measured were assumed to have the same end points as faster reactions; several results from previous work suggest that the reaction end points are not significantly affected by substrate or ribozyme identity (*16*−*18*; G.J.N. and D.H., unpublished results).

Kinetic Constants. $(k_{cat}/K_m)^S$ represents the second-order rate constant for reaction of E^G + S → products. Under "$(k_{cat}/K_m)^S$ conditions", most of S is not bound to E^G and the rate constant is linearly proportional to [E] with a slope that equals $(k_{cat}/K_m)^S$. The rate constants were measured with 2 mM G [K_d^G = 1 mM (*19*, *20*)], and typically, three concentrations of E were used for each $(k_{cat}/K_m)^S$ determination. k_3 represents the third-order rate constant for reaction of E + G + S → products. Under these conditions, the rate constant for reaction of S at a given concentration of G is linearly proportional to [E] with a slope that equals k_3[G]. Values of k_3 were obtained for reaction of $CCC\underline{mU}CUA_5$ with WT ribozyme using 20 μM G [K_d^G = 1 mM (*19*, *20*)] and three concentrations of E.

Estimation of Errors. Rate constants varied typically by ±20% in independent experiments. The values of K_{dock}^{rel} were calculated from the ratio of individual $(k_{cat}/K_m)^S$ values (eq 2), so the error limits for K_{dock}^{rel} were conservatively estimated as the sum of the error limits for the individual $(k_{cat}/K_m)^S$ values; i.e., ±40% (∼2-fold variation).

RESULTS

Specific Tertiary Interactions Are Not Disrupted upon Shortening the Oligonucleotide Substrate. Shortening the standard oligonucleotide substrate, $CCCUCUA_5$, to $UCUA_5$ retains all of the P1 functional groups known to be involved in tertiary interactions, yet the equilibrium constant for P1 docking, K_{dock}, is reduced by ∼200-fold (Figure 1; *11*). A simple model for the weaker docking of $UCUA_5$ is that the tertiary interaction made by the 2′-OH group of G25 is disrupted (Figure 2A,B). This could arise because the base pair with G25 that is present in the standard P1 duplex is broken in the P1 duplex formed with $UCUA_5$, and this base pair may be required for proper positioning of the 2′-OH group with respect to functional groups in the ribozyme core. To test this hypothesis, we compared the equilibrium for docking of $UCUA_5$ with the wild-type (WT) ribozyme to

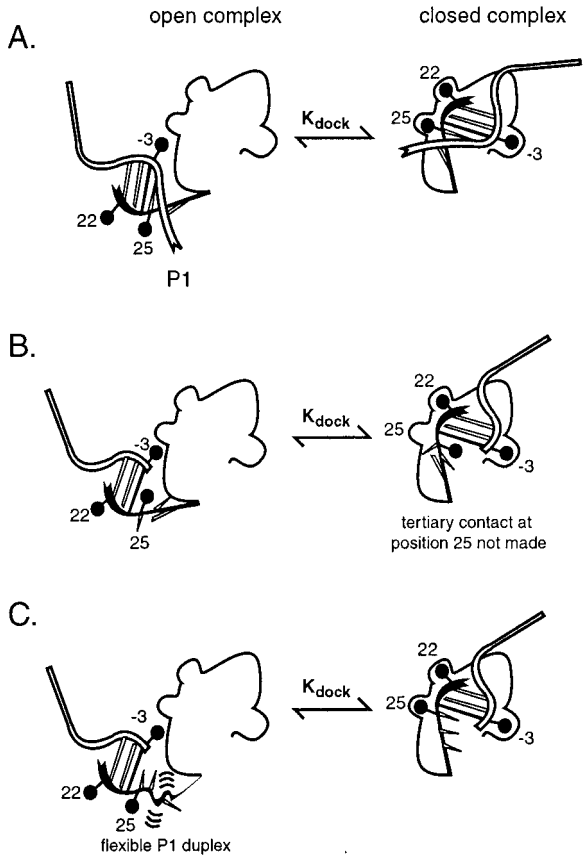

FIGURE 2: Models to account for the weaker docking of $UCUA_5$. The 2′-OH groups at G22, −3U, and G25 are depicted schematically by the black circles and the ribozyme active site by the outline. (A) The full-length substrate forms a rigid P1 duplex that docks into the active site. (B) Model in which disruption of the base pair with G25 in the P1 duplex formed with $UCUA_5$ disrupts the positioning of the 2′-OH group of G25 within the active site. (C) Model in which shortening the oligonucleotide substrate increases the flexibility of the P1 duplex in the open complex, thereby increasing the entropic cost of docking.

the equilibrium for docking with a modified ribozyme that has the 2′-OH group of G25 replaced with a 2′-H (dG25).

The weak binding of $UCUA_5$ to E precludes direct measurement of its binding affinity (*11*). K_{dock} values for $UCUA_5$ were therefore obtained by an indirect approach used previously. In this approach, $(k_{cat}/K_m)^S$, the second-order rate constant for the reaction $E^G + S \rightarrow$ products, estimated P1 duplex stabilities, and the rate constant for the chemical step are used to calculate K_{dock} (*11*). The reaction of E^G with S involves three individual steps: P1 duplex formation, P1 docking, and chemical cleavage (Figure 1A; *7*, *8*, *16*, *21*). We have previously shown that $(k_{cat}/K_m)^S$ for reaction of $UCUA_5$ with the WT ribozyme is limited by the chemical step between pH 5 and 7 (*11*). Under these conditions, the value of $(k_{cat}/K_m)^S$ relates to the equilibrium dissociation constant of the open complex, K_d^{IGS}, K_{dock}, and the rate constant for the chemical step, k_{chem}, according to eq 1a, which is derived from Figure 1A.

$$(k_{cat}/K_m)^S = k_{chem}K_{dock}/K_d^{IGS} \quad (1a)$$

$$K_{dock} = (k_{cat}/K_m)^S K_d^{IGS}/k_{chem} \quad (1b)$$

Rearrangement of eq 1a gives the expression for K_{dock} (eq 1b), and K_{dock} values for the WT and dG25 ribozyme can then be compared by use of eq 2, which is obtained from eq 1b. In eq 2, P1 refers generically to the duplex formed between WT ribozyme and $UCUA_5$ or $CCCUCUA_5$, and modified P1 refers to the corresponding P1 duplex having a 2′-H or other substitution.

$$K_{dock}^{rel} = (k_{cat}/K_m)^{S,rel} K_d^{IGS,rel}/k_{chem}^{rel} \quad (2)$$

where

$$K_{dock}^{rel} = K_{dock}^{(P1)}/K_{dock}^{(modified\ P1)}$$

$$(k_{cat}/K_m)^{S,rel} = (k_{cat}/K_m)^{S(P1)}/(k_{cat}/K_m)^{S(modified\ P1)}$$

$$K_d^{IGS,rel} = K_d^{IGS(P1)}/K_d^{IGS(modified\ P1)}$$

$$k_{chem}^{rel} = k_{chem}^{(P1)}/k_{chem}^{(modified\ P1)}$$

In eq 2, the value of k_{chem}^{rel} was taken to be 1 because k_{chem} is similar for the full-length substrate and $UCUA_5$ and k_{chem} for the full-length substrate is not affected by the dG25 modification (*11*, *14*, *18*). The value of $K_d^{IGS,rel}$ was also taken to be 1 because, in the context of the P1 duplex formed with $UCUA_5$, the 2′-H substitution is in an unpaired region of the IGS. Table 1 gives the K_{dock}^{rel} value obtained from the measured values of $(k_{cat}/K_m)^S$. Substituting the 2′-OH of G25 with a 2′-H destabilizes docking of $UCUA_5$ by 43-fold ($\Delta\Delta G_{tert} = 2.4$ kcal/mol), suggesting that the 2′-OH group provides substantial tertiary stabilization in the context of bound $UCUA_5$. Further, the tertiary stabilization provided by the 2′-OH at G25 is the same, within error, in the context of bound $UCUA_5$ and $CCCUCUA_5$ (Table 1, $\Delta\Delta G_{tert} = 2.4$ vs 2.5 kcal/mol for $UCUA_5$ and $CCCUCUA_5$, respectively). Thus, the tertiary interaction made by the 2′-OH of G25 is maintained upon shortening of the substrate. This suggests that the G25 2′-OH group is adequately positioned by its proximity to the portion of the P1 duplex that is formed and, presumably, by the ability of the G25 base to stack on the duplex (Figure 1B).

We next tested whether the other major tertiary interactions are maintained upon docking of $UCUA_5$. In individual experiments, the exocyclic amino group at G22 was replaced with a hydrogen atom to give inosine (I22), and the 2′-OH groups at G22 and −3U were replaced with 2′-H groups (Figure 1B). The effects of each of these substitutions on K_{dock} were measured as above; these substitutions were also assumed not to affect the chemical step ($k_{chem}^{rel} = 1$ in eq 2) because k_{chem} for the full-length substrate is not affected by the modifications (*14*, *18*). The effects of these substitutions on K_d^{IGS} ($K_d^{IGS,rel}$) have been measured previously for the standard P1 duplex (*22*). These effects were assumed to be the same for the P1 duplex with $UCUA_5$ because effects of 2′-OH → H and G → I substitutions in model duplexes are largely independent of neighboring residues (*22*−*24*). The K_{dock}^{rel} values for the above substitutions are summarized in Table 1 and the $\Delta\Delta G_{tert}$ values obtained are compared to those obtained previously in the context of the P1 duplex formed with $CCCUCUA_5$. Each of the substitutions that destabilizes docking of $CCCUCUA_5$ also destabilizes docking of $UCUA_5$. This suggests that the tertiary interactions made by the exocyclic amino group of G22 and the 2′-OH

Table 1: Comparison of the Tertiary Stabilization Provided by Functional Groups on the P1 Duplex Formed with $UCUA_5$ and $CCCUCUA_5$[a]

	$UCUA_5$						$CCCUCUA_5$
P1 duplex	$(k_{cat}/K_m)^S$ [b] $M^{-1}min^{-1}$	$(k_{cat}/K_m)^{S,rel}$ [c]	$K_d^{IGS,rel}$ [d]	k_{chem}^{rel} [e]	K_{dock}^{rel} [f]	$\Delta\Delta G_{tert}$ [g] (kcal/mol)	$\Delta\Delta G_{tert}$ [g,h] (kcal/mol)
5′(CCC)UCUA$_5$ GGG AGG	2.9×10^4	(1)	(1)	(1)	(1)	(0)	(0)
5′(CC C)UCUA$_5$ GGdG AGG	6.8×10^2	43	1 [i]	1	43	**2.4**	**2.5**
5′(CCC)UC UA$_5$ GGG AGdG	4.9×10^2	59	0.27 [j]	1	16	**1.8**	**3.1**
5′(CCC)UCUA$_5$ GGG AGI	6.2×10^2	47	0.33 [j]	1	16	**1.8**	**2.1**
5′(CCC)dUCUA$_5$ GGG AGG	3.8×10^2	76	0.26 [j]	1	20	**1.9**	**0.9**

[a] Conditions: 50 °C, 50 mM sodium MES, pH 6.6, and 10 mM $MgCl_2$. [b] $(k_{cat}/K_m)^S$ is the second-order rate constant for the reaction $E^G + S \rightarrow$ products (see Materials and Methods). [c] $(k_{cat}/K_m)^{S,rel} = (k_{cat}/K_m)^{S(P1)}/(k_{cat}/K_m)^{S(modified\ P1)}$; P1 refers to the P1 duplex formed between $UCUA_5$ or $CCCUCUA_5$ and the WT ribozyme, and modified P1 refers to a P1 duplex having a 2′-deoxy or G22 → I substitution. [d] $K_d^{IGS,rel} = K_d^{IGS(P1)}/K_d^{IGS(modified\ P1)}$; K_d^{IGS} is the equilibrium dissociation constant for the open complex (Figure 1A). [e] $k_{chem}^{rel} = k_{chem}^{(P1)}/k_{chem}^{(modified\ P1)}$; k_{chem} is the rate constant for reaction from the closed complex (Figure 1A). As described in the Results, k_{chem}^{rel} was assumed to be equal to 1 because $k_{chem}^{rel} = 1$ in the context of the full-length substrate. [f] $K_{dock}^{rel} = K_{dock}^{(P1)}/K_{dock}^{(modified\ P1)}$ and was determined from eq 2 as described in the Results; $K_{dock}^{rel} > 1$ indicates a destabilizing effect from the modification. [g] $\Delta\Delta G_{tert} = RT \ln (K_{dock}^{rel})$; $R = 0.00198$ kcal mol^{-1} K^{-1} and $T = 323$ K (50 °C). $\Delta\Delta G_{tert} > 0$ indicates a destabilizing effect from the modification. [h] $\Delta\Delta G_{tert}$ was taken from previous measurements (*18, 22*). [i] $K_d^{IGS,rel}$ was assumed to be equal to 1 because the 2′-H substitution is in an unpaired region of the IGS (see Results). [j] $K_d^{IGS,rel}$ values were assumed to be the same as those previously determined in the context of the full-length P1 duplex [(*18, 22*); see Results].

groups of G22 and −3U are maintained upon shortening of the substrate.

Whereas the 2′-OH group of G25 and the exocyclic amino group of G22 provide similar tertiary stabilization in the context of $CCCUCUA_5$ and $UCUA_5$, the 2′-OH groups of −3U and G22 contribute ∼1 kcal/mol more and less, respectively, in the context of the shorter substrate. These differences do not account for the 3.4 kcal/mol destabilization observed for docking of $UCUA_5$ relative to $CCCUCUA_5$ and may be due, in part, to small errors in the estimation of $K_d^{IGS,rel}$ and k_{chem}^{rel} values as well as small differences in the precise positioning of the two P1 duplexes within the active site. Although the tertiary interactions made by the 2′-OH groups of −2C, G23, and G26 (Figure 1B) were not tested herein, replacing these 2′-OH groups with 2′-H groups destabilizes docking of the full-length substrate by only 0.4, 0.8, and 0.8 kcal/mol, respectively, not enough to account for the observed 3.4 kcal/mol destabilization (*22*). Further, there is no indication of any additional tertiary interactions involving bases and phosphate groups (*25*; J. A. Piccirilli, personal communication). Together, the results suggest that the P1 duplex formed with $UCUA_5$ makes the same tertiary interactions as the full-length P1 duplex and that the weaker docking of $UCUA_5$ does not arise from disruption of these tertiary interactions.

Shortening the Oligonucleotide Substrate Can Decrease Tertiary Interaction Specificity. To test whether shortening the oligonucleotide substrate affects the specificity of tertiary interactions, 2′-OH groups at two positions, G25 and −3U, were replaced with the larger 2′-methoxy group. For the shortened substrates, the effects of the methoxy substitution on docking (K_{dock}^{rel}) could be obtained from eqs 1 and 2 as above because $(k_{cat}/K_m)^S$ is limited by the chemical step (*11*). The value of $K_d^{IGS,rel}$ in eq 2 was taken to be 1 because 2′-methoxy substitutions do not significantly alter the stability of model RNA/RNA duplexes (*10, 15, 26, 27*; G.J.N. and D.H., unpublished results). It was also assumed that the methoxy substitutions do not alter the rate of the chemical step ($k_{chem}^{rel} = 1$ in eq 2).[2]

For reaction of $CCCUCUA_5$ with the mG25 ribozyme, the decreased value of $(k_{cat}/K_m)^S$ relative to that for reaction of $CCCUCUA_5$ with the WT ribozyme and the observed guanosine dependence suggested that the chemical step was rate-limiting, thereby allowing the use of eq 1b (data not shown). However, $(k_{cat}/K_m)^S$ for reactions of $CCCUCUA_5$ and $CCC\underline{mU}CUA_5$ with WT ribozyme is not limited by the chemical step (*7, 8, 11, 16, 28, 29*; G.J.N. and D.H., unpublished results) and so the value of $(k_{cat}/K_m)^S$ for these reactions does not equal $(k_{chem}K_{dock}/K_d^{IGS})$ as in eq 1a. This precluded the use of eq 2 to obtain K_{dock}^{rel}. The value of K_{dock}^{rel} was therefore obtained by an alternate approach in which the value of $(k_{chem}K_{dock}/K_d^{IGS})$ was explicitly defined as the ratio k_{app}:

$$k_{app}^{(P1)} = k_{chem}^{(P1)}K_{dock}^{(P1)}/K_d^{IGS(P1)} \quad (3a)$$

$$k_{app}^{(modified\ P1)} = k_{chem}^{(modified\ P1)}K_{dock}^{(modified\ P1)}/K_d^{IGS(modified\ P1)} \quad (3b)$$

For the reasons stated above, k_{chem}^{rel} and $K_d^{IGS,rel}$ were assumed to equal 1. Under these conditions

$$K_{dock}^{rel} = k_{app}^{(P1)}/k_{app}^{(modified\ P1)} \quad (4)$$

[2] This assumption was made because changing the 2′-OH groups at these positions has no effect on k_{chem} for the full-length substrate and because the positions of substitution are three residues removed from the site of chemical cleavage (*14, 15, 18*). If this assumption does not hold, the discrimination against 2′-methoxy substitutions would reflect the specificity of tertiary interactions in the chemical transition state. This would not affect the overall conclusion from this section that shortening the oligonucleotide substrate can reduce the specificity of tertiary interactions.

Table 2: Energetic Effects of 2′-Methoxy Substitutions on the Docking of $UCUA_5$ and $CCCUCUA_5$[a]

P1 duplex	$(k_{chem} \times K_{dock} / K_d^{IGS})$ [b] $M^{-1}min^{-1}$	$(k_{chem} \times K_{dock} / K_d^{IGS})^{(rel)}$ [c]	$K_d^{IGS,rel}$ [d]	k_{chem}^{rel} [e]	K_{dock}^{rel} [f]	$\Delta\Delta G_{tert}$ [g] (kcal/mol)
5′UCUA$_5$ GGGAGG	2.9×10^4	(1)	(1)	(1)	(1)	**(0)**
5′UCUA$_5$ GGmGAGG	280	104	1	1	104 [h]	**3.0**
5′mUCUA$_5$ GGG AGG	150	193	1	1	193 [h]	**3.4**
5′CCCUCUA$_5$ GGGAGG	1.8×10^{11} [i]	(1)	(1)	(1)	(1)	**(0)**
5′CC CUCUA$_5$ GGmGAGG	6.5×10^6	2.8×10^4	1	1	2.8×10^4 [j]	**6.5**
5′CCCmUCUA$_5$ GGG AGG	4.1×10^8 [k]	439	1	1	439 [j]	**3.9**

[a] Conditions: 50 °C, 50 mM sodium MES, pH 6.6, and 10 mM $MgCl_2$. [b] For all P1 duplexes except those indicated, $(k_{chem}K_{dock}/K_d^{IGS})$ equals $(k_{cat}/K_m)^S$, the second-order rate constant for the reaction $E^G + S \rightarrow$ products as described in the Results. $(k_{cat}/K_m)^S$ was obtained as described in Materials and Methods. [c] $(k_{chem}K_{dock}/K_d^{IGS})^{(rel)} = (k_{chem}K_{dock}/K_d^{IGS})^{(P1)}/(k_{chem}K_{dock}/K_d^{IGS})^{(modified\ P1)}$; P1 refers to the P1 duplex formed between $UCUA_5$ or $CCCUCUA_5$ and the WT ribozyme, and modified P1 refers to a P1 duplex having a 2′-methoxy substitution at G25 or −3U. [d] $K_d^{IGS,rel} = K_d^{IGS(P1)}/K_d^{IGS(modified\ P1)}$; K_d^{IGS} is the equilibrium dissociation constant for the open complex and was assumed to equal 1 for the 2′-methoxy modifications as described in the Results. [e] $k_{chem}^{rel} = k_{chem}^{(P1)}/k_{chem}^{(modified\ P1)}$; k_{chem} is for reaction from the closed complex (Figure 1A). As described in the Results, k_{chem}^{rel} was assumed to equal 1. [f] $K_{dock}^{rel} = K_{dock}^{(P1)}/K_{dock}^{(modified\ P1)}$. $K_{dock}^{rel} > 1$ indicates a destabilizing effect from the 2′-methoxy modification. [g] $\Delta\Delta G_{tert} = RT \ln (K_{dock}^{rel})$; $R = 0.00198$ kcal mol^{-1} K^{-1} and $T = 323$ K (50 °C). $\Delta\Delta G_{tert} > 0$ indicates a destabilizing effect from the 2′-methoxy modification. [h] Obtained from eq 2. [i] $(k_{chem}K_{dock}/K_d^{IGS})$ was obtained from the previously determined k_{chem} value of 200 min^{-1}, K_{dock} value of 63, and K_d^{IGS} value of 7.0×10^{-8} M (*11, 15*). [j] Obtained from eq 4. [k] $(k_{chem}K_{dock}/K_d^{IGS})$ was obtained from k_3, the measured third-order rate constant for reaction of $E + G + S \rightarrow$ products, which equals $(k_{chem}K_{dock})/(K_d^{IGS}K_d^G)$, using the previously determined value of 1×10^{-3} M for K_d^G (*19, 20*) (see eq 5).

In eq 4, the value of $k_{app}^{(P1)}$, which is for the reaction of $CCCUCUA_5$ with WT ribozyme, was obtained from the previously determined values of k_{chem}, K_d^{IGS}, and K_{dock} ($k_{app}^{(P1)} = 1.8 \times 10^{11}$ M^{-1} min^{-1}; Table 2) (*11, 15*).

For reaction of $CCCmUCUA_5$ with WT ribozyme, $k_{app}^{(modified\ P1)}$ was obtained by measuring k_3, the third-order rate constant for the reaction $E + G + S \rightarrow$ products. Under conditions of rate-limiting chemical cleavage, k_3 relates to k_{chem}, K_{dock}, K_d^{IGS}, and the equilibrium constant for dissociation of guanosine from E^G, K_d^G, according to

$$k_3 = k_{chem}K_{dock}/(K_d^{IGS})K_d^G \qquad (5a)$$

which was derived from the following reaction:[3]

$$E + G + S \overset{K_d^G}{\rightleftharpoons} E^G + S \overset{K_d^{IGS}}{\rightleftharpoons} (E^G{\cdot}S)_o \overset{K_{dock}}{\rightleftharpoons} (E^G{\cdot}S)_c \xrightarrow{k_{chem}} \text{products}$$

The previously measured value of K_d^G was then used to obtain the value of $k_{app}^{(modified\ P1)}$ from

$$k_{app}^{(modified\ P1)} = k_{chem}K_{dock}/K_d^{IGS} = k_3K_d^G \qquad (5b)$$

[Table 2 (*19, 20*)]. For reaction of $CCCUCUA_5$ with the mG25 ribozyme, $k_{app}^{(modified\ P1)}$ equals $(k_{cat}/K_m)^S$ as in eq 1a above because this reaction is limited by the chemical step. The $k_{app}^{(P1)}$ and $k_{app}^{(modified\ P1)}$ values obtained above were then used in eq 4 to obtain K_{dock}^{rel} values for the 2′-methoxy substitutions in the context of the full-length substrate.

Table 2 summarizes the K_{dock}^{rel} values obtained in the context of the full-length and shortened oligonucleotide substrates. The 2′-methoxy substitution at G25 destabilizes docking of the full-length substrate by $\sim 3 \times 10^4$-fold, whereas the same substitution destabilizes docking of $UCUA_5$ by only 10^2-fold (Table 2, $\Delta\Delta G_{tert} = 6.5$ vs 3.0 kcal/mol, respectively). In contrast, the 2′-methoxy substitution at −3U has comparable destabilizing effects in the context of the full-length and shortened substrates (Table 2, $\Delta\Delta G_{tert} = 3.9$ and 3.4 kcal/mol for $CCCUCUA_5$ and $UCUA_5$, respectively).

DISCUSSION

The *Tetrahymena* ribozyme's oligonucleotide substrate, $CCCUCUA_5$, binds to the ribozyme in two steps: P1 duplex formation with the ribozyme's internal guide sequence to give the open complex is followed by docking of the duplex into tertiary interactions with the active site to give the closed complex (Figure 1). Previous work suggests that shortening the substrate to $UCUA_5$ destabilizes the closed complex by 200-fold (3.4 kcal/mol) (*11*). One model to account for the weaker docking of $UCUA_5$ entails disruption of the tertiary interaction made by the 2′-OH at G25, as this residue is no longer base-paired in the P1 duplex formed with $UCUA_5$ (Figure 2B). However, the results here demonstrate that this and other tertiary interactions are maintained upon shortening of the substrate. This strongly suggests that the weaker docking of $UCUA_5$ is not due to disruption of specific tertiary interactions. The weaker docking is therefore most simply explained by the decreased rigidity of the open complex

[3] In this reaction, $(E^G{\cdot}S)_c$ refers to the closed complex and $(E^G{\cdot}S)_o$ to the open complex. Under conditions of rate-limiting chemical cleavage there is sufficient time to establish an equilibrium population of $(E^G{\cdot}S)_c$, and k_3, the rate constant for the reaction $E + G + S \rightarrow$ products, relates to the rate and equilibrium constants in this reaction according to eq 5a in the text. Although binding of G is shown to precede binding of S in the reaction scheme shown in the text, the alternate pathway in which S binds before G also gives the same equation because $(E^G{\cdot}S)_c$ is in equilibrium with free E, S, and G prior to rate-limiting chemical cleavage.

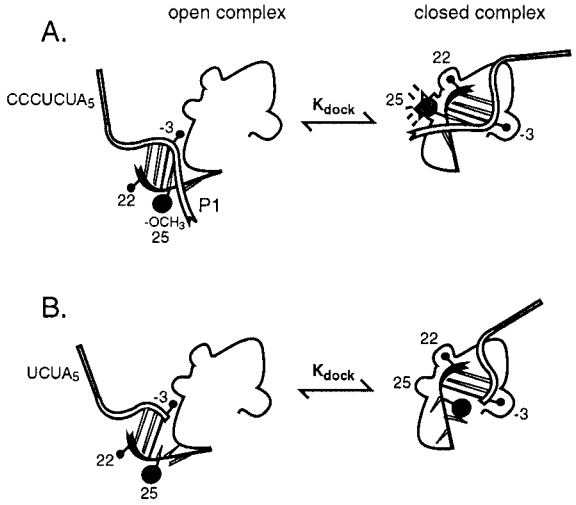

FIGURE 3: Model for the decreased discrimination against the 2′-methoxy group at G25 in the context of the P1 duplex with $UCUA_5$. The 2′-methoxy group at G25 is depicted schematically by the large black circle. (A) In the context of the full-length P1 duplex, base-pairing of G25 limits rearrangement of the 2′-methoxy group at this position, resulting in unfavorable steric interactions. (B) With bound $UCUA_5$, G25 is not base-paired and its 2′-methoxy group can readily rearrange to avoid the steric clashes.

formed with $UCUA_5$ (Figure 2C). P1 duplex formation with $UCUA_5$ leaves three IGS residues unpaired, which are expected to increase the flexibility of the open complex and thereby increase the entropic cost of docking the P1 duplex relative to the standard P1 duplex with all six base pairs.[4] It is also possible that additional conformational rearrangements are allowed in the open complex with $UCUA_5$, giving an additional energetic penalty for docking. The large energetic advantage (3.4 kcal/mol) gained from using the more rigid open complex containing the standard full-length P1 duplex underscores and provides a quantitative measure for the role of duplex rigidity in forming stable tertiary structures.

The results further suggest that presentation of functional groups in the context of a duplex can substantially increase interaction specificity. In the context of the P1 duplex formed with $UCUA_5$, the 2′-OH group at G25 stabilizes the closed complex by 3.0 kcal/mol more than the bulkier 2′-methoxy group (Table 2). In the context of the full-length P1 duplex formed with $CCCUCUA_5$, the specificity for this 2′-OH group is increased by 3.5 kcal/mol. These observations are most simply explained by the greater conformational restriction of a residue within a base pair versus a single strand (Figure 3). Substitution of the 2′-OH group at G25 with the bulkier 2′-methoxy group is expected to result in unfavorable steric interactions with nearby active-site groups. In the context of the standard P1 duplex, the 2′-methoxy group cannot readily avoid the unfavorable interactions because rearrangement of the methoxy group is hindered by the constraints imposed by duplex geometry. Avoiding the unfavorable interactions may require alteration of the position of the rest of the P1 duplex, unpairing of the duplex, or local disruption of the ribozyme core. In contrast, in the P1 duplex formed with $UCUA_5$, the G25 residue is not base-paired, so its 2′-methoxy group has greater conformational freedom. This allows the 2′-methoxy group to rearrange with a smaller energetic penalty. Indeed, in the context of $UCUA_5$, the 2′-methoxy substitution at G25 has the same destabilizing effect, within error, as the much smaller 2′-H substitution, consistent with an ability of the 2′-methoxy group to move away from unfavorable steric interactions without a significant energetic cost (Tables 1 and 2, $\Delta\Delta G_{tert} = 2.4$ vs 3.0 kcal/mol for 2′-H vs 2′-methoxy substitution, respectively).

The model in Figure 3 can also explain why a 2′-methoxy substitution at −3U destabilizes the closed complex to comparable extents in the context of $UCUA_5$ and $CCCUCUA_5$: the −3U residue is base-paired in both the P1 duplexes and hence the 2′-methoxy group at this position has similar constraints for rearrangement. Some of the modified bases and sugars present on duplexes in ribosomal and other RNAs may analogously aid correct folding by destabilizing alternative nonnative interactions.

In summary, the rigidity provided by the P1 duplex greatly aids the *Tetrahymena* ribozyme's oligonucleotide substrate in forming stable and specific tertiary interactions. Presentation of functional groups in the context of secondary structural elements may be a general strategy employed by RNA to facilitate formation of stable and specific tertiary structures.

[4] In principle, comparison of the temperature dependencies of K_{dock} for $UCUA_5$ and $CCCUCUA_5$ can provide information about effects that are entropic in origin ($\Delta S°'$). However, observed values of $\Delta S°'$ include unpredictable effects from additional structural rearrangements and solvent reorganization that can obscure the contributions from an increase in chain mobility (*30*).

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