Enhancement of RNA/Ligand Association Kinetics via an Electrostatic Anchor

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Supporting Information

ABSTRACT: The diverse biological processes mediated by RNA rest upon its recognition of various ligands, including small molecules and nucleic acids. Nevertheless, a recent literature survey suggests that RNA molecular recognition of these ligands is slow, with association rate constants orders of magnitude below the diffusional limit. Thus, we were prompted to consider strategies for increasing RNA association kinetics. Proteins can accelerate ligand association via electrostatic forces, and here, using the Tetrahymena group I ribozyme, we provide evidence that electrostatic forces can accelerate RNA/ligand association. This RNA enzyme (E) catalyzes cleavage of an oligonucleotide substrate (S) by an exogenous guanosine (G) cofactor. The G 2′- and 3′-OH groups interact with an active site metal ion, termed MC, within E:S·G, and we perturbed each of these contacts via –NH3+ substitution. New and prior data indicate that G(2′NH3+) and G(3′NH3+) bind as strongly as G, suggesting that the –NH3+ substituents of these analogues avoid repulsive interactions with MC and make alternative interactions. Unexpectedly, removal of the adjacent –OH via –H substitution to give G(2′H,3′NH3+) and G(2′NH3+,3′H) enhanced binding, in stark contrast to the deleterious effect of these substitutions on G binding. Pulse-chase experiments indicate that the –NH3+ moiety of G(2′H,3′NH3+) increases the rate of G association. These results suggest that the positively charged –NH3+ group can act as a molecular “anchor” to increase the residence time of the encounter complex and thereby enhance productive binding. Electrostatic anchors may provide a broadly applicable strategy for the development of fast binding RNA ligands and RNA-targeted therapeutics.
attract oppositely charged ligands to provide binding rate constants at and in excess of the diffusion “limit.”25−30 Electrostatic fields are also presumably critical for enabling one-dimensional diffusion of proteins along DNA and thus efficient searches for specific recognition sequences and damaged DNA bases.31−33

For RNA, the negative charge on its phosphodiester backbone creates a powerful electrostatic potential for binding to cationic ligands. These electrostatics are most broadly manifest in the ion atmosphere that surrounds RNA molecules,34−36 a preponderance of cations that contribute to overall neutralization as predicted for polyelectrolytes such as RNA and DNA from simple electrostatic theories.37 Beyond the general attraction of positively charged ions, RNA often binds tightly to cationic small molecules, including polyamines and aminoglycoside antibiotics (e.g., refs 39−44), as well as peptide sequences rich in acidic residues (e.g., lysine and arginine),45 as well as peptide sequences rich in acidic residues (e.g., lysine, arginine),45 with affinities in the micromolar and sub-micromolar range. Many of these charged ligands bind to several RNAs, and such broad specificity may reflect RNA’s inherent tendency to assume stable alternative structures46,47 that can make favorable electrostatic contacts with cationic ligands.

In the course of exploring a paradoxical observation for molecular recognition by the Tetrahymena group I ribozyme, we uncovered an electrostatic enhancement of RNA/ligand association. As described below, our results led to a recognition model via an electrostatic “binding anchor” to increase the efficiency and rate of binding. This approach may be of value in the design of RNA ligands in engineering and therapeutics.

## MATERIALS AND METHODS

**Materials.** L-21 Scfl ribozyme (E) was transcribed and gel-purified according to reported procedures.48 Care was taken to avoid RNA damage from ultraviolet shadowing, as previously described.49 Guanosine (G) was purchased from Sigma-Aldrich (St. Louis, MO) with a purity of ≥98%, and 3′-amino-2′-deoxyguanosine [G(2′N)] and 3′-amino-2′-deoxyguanosine [G(2′N,3′H)] were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and were of the highest purity commercially available (≥98%). 2′-Amino-3′-deoxyguanosine [G(2′N,3′H)] was a gift from J. W. Szostak (Harvard University, Cambridge, MA). The oligonucleotide substrates, CCCUCUA (rSA) and CCCUCUUA (−1d,rSA), were purchased from Integrated DNA Technologies (Redwood City, CA), 5′−32P-radioabeled using [γ−32P]ATP (MP Biomedicals, Santa Ana, CA) and T4 polynucleotide kinase (New England Biolabs, Ipswich, MA) according to the manufacturer’s protocol, and gel-purified as previously described.51 Buffers and salts were purchased from Sigma-Aldrich. All nonradioactive reagents were passed through a 0.2 μm sterile syringe filter (Corning, Corning, NY) prior to use.

**General Reaction Conditions.** Single-turnover reactions, with ribozyme in excess of radiolabeled substrate, were measured at 30 °C in the presence of MgCl2 (10−100 mM) and 50 mM buffer. The following buffers were used in ribozyme-catalyzed reactions: sodium acetate, pH 5.0−5.5; NaMES, pH 6.1−6.7; NaMOPS, pH 7.1; NaEPPS, pH 7.7−8.2; and NaCHES, pH 8.7−9.7.

Reactions were carried out and analyzed according to reported procedures.52 Ribozyme was allowed to fold in 10 mM MgCl2 and 50 mM buffer at 50 °C for 30 min and then cooled to room temperature. For reactions above pH 8.0, the folding step was performed in 25 mM NaMES (pH 6.7) to avoid ribozyme degradation. Following the folding step, ribozyme was diluted 20-fold in reaction tubes containing the desired concentrations of divalent metal ion (MgCl2 and MnCl2), buffer, and G/G analogue. After a 5 min incubation at 30 °C, reactions were initiated by the addition of a labeled substrate (<0.1 nM). At specified times, six 2 μL aliquots of the reaction mixture were removed from the 20 μL reaction mixture and added to a 4 μL quench solution containing 90% formamide, 50 mM EDTA, 0.01% bromphenol blue, and 0.01% xylene cyanol. The substrate and product were separated by electrophoresis on a 20% polyacrylamide gel containing 7 M urea, 100 mM Tris, 83 mM boric acid, and 1 mM EDTA. The ratio of substrate to product was quantitated through phosphorimager analysis (GE Healthcare) with TotalLab (TotalLab Ltd.).

Reactions were followed for ≥3t1/2 except for very slow reactions. First-order fits (R2 > 0.95) to the data points, with end points of ≥90%, were obtained (KaleidaGraph, Synergy Software). The slow reactions were typically linear for up to 20 h, and an end point of 95% was assumed to obtain observed rate constants from the initial rates.

**Measurement of Affinities of G for E−S.** The binding affinity of G for the E−S complex was determined by measuring the observed rate of cleavage (kobs) of 5′-end-labeled rSA (or −1d,rSA) at different G concentrations under conditions where E is saturating with respect to S (|E| = 50 nM; Kd ~ 1 nM).52 kobs was plotted as a function of G concentration, and the data were fit to eq 1 to obtain K1/2:

\[
k_{\text{obs}} = \frac{k_{\text{max}[G]}}{[G] + K_{1/2}^{G}}
\]

To ensure that K1/2G is equal to KdS, we used the rSA substrate at pH <6 as prior data indicate that the chemical step is rate-limiting at and below this pH.53−55 Above pH 6, we used the −1d,rSA substrate that contains a 2′-H substitution at the U(−1) position that renders the chemical step rate-limiting.53−55

**Measurement of Affinities of G(3′N), G(2′H,3′N), and G(2′N,3′H) for E−S.** We did not observe any detectable cleavage activity for G(3′N), G(2′H,3′N), and G(2′N,3′H) and thus measured binding of these analogues to E−S through competitive inhibition of the reaction E−S + G → products. Experiments were performed using 5′-end-labeled rSA (or −1d,rSA) with E saturating with respect to S (see above) and with sub saturating G (|G| = 30 μM; Kd ~ 100 μM).56 The concentration of inhibitor, Gx, was varied, and the inhibition constant, Kx report the affinity of Gx for E−S was determined via eq 2:

\[
k_{\text{obs}} = \frac{k_{\text{max}K_{x}}}{[G_{x}]+K_{x}}
\]

where kobs is the observed rate of cleavage of rSA (or −1d,rSA) and kmax is kobs in the absence of an inhibitor.

**Monitoring Binding of G(3′NH3+)x, G(2′H,3′NH3+)x, and G(2′NH3+,3′H) to E−S.** The −NH2 groups of G(3′N), G(2′H,3′N), and G(2′N,3′H) can ionize to form the corresponding −NH3+ species. To ensure that we were monitoring the −NH3+ forms of these analogues, we measured the binding of each analogue at various pH values (Figures S1−S3). For all guanosine analogues bearing the −NH2 moiety, we observed that the level of binding to E−S increased.
with a decrease in pH and did not vary below pH 6. The data were fit to an equation for binding of the $-\text{NH}_3^+$ and $-\text{NH}_3\text{H}^+$ forms of G(3'N), G(2'H,3'N), and G(2'N,3'H) (Figures S1–S3) to obtain binding constants for the neutral and protonated forms of these analogues.

**Pulse–Chase Assay for Measuring $k_{\text{off}}$ for G-(2'H,3'NH$_3^+$).** To measure the dissociation rate constant ($k_{\text{off}}$) for G(2'H,3'NH$_3^+$), we carried out a pulse–chase assay. In a typical experiment, 5'-end-labeled rSA ([*S] $< 1$ nM) was incubated with saturating ribozyme ([E] = 100 nM; $K_d \approx 1$ nM) and saturating G(2'H,3'NH$_3^+$) ([G(2'H,3'N)] = 350 nM; $K_d^{(G(2'H,3'N))} = 94$ nM (Table S2)) at various times ($t_1$ = 1, 10, and 60 min) in 50 mM sodium acetate (pH 5.5) and 10 mM Mg$_2^+$. After $t_1$, the reaction mixture was diluted 10-fold with a chase solution containing 2 mM G, 50 mM sodium acetate (pH 5.5), and 10 mM Mg$_2^+$. Following addition of the chase, six 2 μL aliquots were withdrawn at various times ($t_2$) and added to a 4 μL quench solution containing 90% formamide, 50 mM EDTA, 0.01% bromophenol blue, and 0.01% xylene cyanol. The substrate and product were separated and analyzed as described above.

**RESULTS**

The *Tetrahymena* group I ribozyme (E) catalyzes cleavage of an oligonucleotide substrate (S) by an exogenous guanosine (G) co-factor. We previously provided biochemical evidence for metal ion interactions between the G 2'- and 3'-OH groups and an active site metal ion termed MC (Figure 1) through assays that replaced each of these −OH groups with an amino (−NH$_2$) moiety,56 and these interactions are consistent with X-ray crystallographic models.57,58 Below we describe the surprising effects of the protonated (−NH$_3^+$) forms of these analogues, G(2'NH$_3^+$) and G(3'NH$_3^+$), on binding to the *Tetrahymena* ribozyme.

Despite the proximity of the G 2’- and 3’-OH groups to an active site Mg$^{2+}$ ion [MC (Figure 1)], prior work showed that replacing the G 2’-OH group with an −NH$_3^+$ moiety [G(2'NH$_3^+$)] does not weaken its binding to the E-S complex.59 This observation is consistent with a model in which the positively charged 2’-NH$_3^+$ group of G(2'NH$_3^+$) interacts with one or more negatively charged phosphoryl groups near the G 2’-moiety. The affinity of G(2'NH$_3^+$) is weakened with an increase in the concentration of the divalent metal ion (Mg$^{2+}$ and Mn$^{2+}$), and the Mn$^{2+}$ concentration dependence of this weakening suggests that MC is responsible for this effect, perhaps via electrostatic repulsion with the 2’-NH$_3^+$ group or by directly competing with G(2'NH$_3^+$) for an interaction with one or more active site phosphoryl groups (Figure 1B).59 To learn more about potential electrostatic interactions in and around this site, we carried out analogous experiments with a 3’-G analogue in which the 3’-OH is replaced with an −NH$_3^+$ [G(3'NH$_3^+$)] (Table 1).

Equilibrium constants for binding of G(3'NH$_3^+$) to E-S were obtained by measuring binding of 3’-aminoguanosine through competitive inhibition of reactions with subsaturating G under single-turnover conditions so that the observed inhibition constant is equivalent to the dissociation constant. We measured binding of the analogue across a pH range to ensure that we were measuring binding of G(3'NH$_3^+$) and not G(3'NH$_3$). As expected, decreasing the pH altered the 3’-aminoguanosine affinity but did not change G binding (Figures S1 and S4). The G(3'NH$_3^+$) data were fit to a model for binding of the −NH$_3^+$ and −NH$_2$ forms of 3’-aminoguanosine to obtain the G(3'NH$_3^+$) affinity.

Equilibrium binding constants for G and G(3'NH$_3^+$) are listed in Table 2, and for comparison, we include the previously reported data for G(2'NH$_3^+$) as well as data for G analogues with −H substitutions at the 2’- or 3’-positions.7 The relative effects of −H and −NH$_3^+$ substitutions on G binding are presented graphically in Figure 2. Deoxy substitution at the 2’- or 3’-positions weakens binding of G by 60- or 260-fold, respectively, consistent with a model in which these −OH groups contact MC in the E-S-G complex56 and the G 2’-OH serves as a hydrogen bond donor.60 While substantially weaker binding of G(2'NH$_3^+$) and G(3'NH$_3^+$) is predicted from the close juxtaposition of the G 2’- and 3’-moieties to MC, respectively, both analogues bind 2-fold stronger than G (Figure 2). These results are consistent with a model in which the 3’-NH$_3^+$ group of G(3'NH$_3^+$) makes a favorable interaction with one or more active site residues, analogous to prior observations with G(2'NH$_3^+$).59

![Figure 1. Model of active site interactions in the E-S-G complex of the *Tetrahymena* ribozyme.](image-url)

(A) Atomic model of interactions made with G (green) and S (black) by E (gray). Dotted lines correspond to metal ion or hydrogen bond interactions. Contacts made between MA and MC (blue) and G and S are colored black, while all other interactions are colored gray. The G 2’- and 3’-OH groups, which are modified in this work, are colored red. The model was obtained through molecular modeling of an X-ray structure of the *Azoarcus* group I ribozyme using constraints from available functional data as described previously (ref 7 and references therein). (B) Schematic of group I active site interactions shown in panel A, with filled circles and hatched lines representing metal ion and hydrogen bond interactions, respectively.
Table 1. Guanosine (G) Analogues Used in This Work

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>G</th>
<th>−OH</th>
<th>OH</th>
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<tr>
<td>G</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>G(2′NH₂)</td>
<td>H</td>
<td>OH</td>
<td>NH₂</td>
<td>OH</td>
</tr>
<tr>
<td>G(3′NH₂)</td>
<td>H</td>
<td>NH₂</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>G(2′NH₂,3′H)</td>
<td>H</td>
<td>H</td>
<td>NH₂</td>
<td>OH</td>
</tr>
<tr>
<td>G(2′H,3′NH₂)</td>
<td>H</td>
<td>NH₂</td>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td>AUGC</td>
<td>AUC</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>AUGC(2′H)</td>
<td>AUC</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
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<tr>
<td>AUGC(3′H)</td>
<td>AUC</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
</tr>
</tbody>
</table>

"The 5′-AUC extension enhances binding of the 3′-terminal G residue via base pairing and stacking interactions (forming P9.0 with the ribozyme) and overcoming assay limitations caused by the limited solubility of G(2′H) and G(3′H) (see ref 7 and references therein).

Table 2. Effects of Deoxy (−H) and Protonated Amino (−NH₃⁺) Substitutions on Binding of G to E-S

<table>
<thead>
<tr>
<th>G Analogue</th>
<th>K_d (µM)</th>
<th>1/K_d(µM)</th>
</tr>
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<tbody>
<tr>
<td>G</td>
<td>127 ± 40</td>
<td>(1.0)</td>
</tr>
<tr>
<td>G(2′NH₂)</td>
<td>63</td>
<td>2.0</td>
</tr>
<tr>
<td>G(2′NH₂,3′H)</td>
<td>11 ± 0.5</td>
<td>11.5</td>
</tr>
<tr>
<td>G(3′NH₂)</td>
<td>52 ± 5</td>
<td>2.4</td>
</tr>
<tr>
<td>G(2′H,3′NH₂)</td>
<td>0.27 ± 0.01</td>
<td>470</td>
</tr>
<tr>
<td>AUGC</td>
<td>0.32 ± 0.06</td>
<td>(1.0)</td>
</tr>
<tr>
<td>AUGC(2′H)</td>
<td>20 ± 3</td>
<td>0.016</td>
</tr>
<tr>
<td>AUGC(3′H)</td>
<td>82 ± 11</td>
<td>0.0035</td>
</tr>
</tbody>
</table>

K_d(µM) = K_d analogue / K_d G, K_dG for G, G(2′NH₂,3′H), G(3′NH₂), and G(2′H,3′NH₂) are from Figures S1–S4 and were measured in the presence of 100 mM Mg²⁺. G(2′NH₂) data were from ref 59 and measured in the presence of 100 mM Mg²⁺. AUGC data were from ref 7 and measured in the presence of 50 mM Mg²⁺.

Figure 2. Binding of G and G analogues to E-S. The relative affinity (1/K_d = K_d analogue / K_d G) refers to the K_d for the G or AUGC analogue relative to G or AUGC. Values of K_d(µM) were obtained from Table 2. The dashed line corresponds to K_d(µM) = 1.

Having observed that G(2′NH₂) and G(3′NH₂) form stable complexes with the ribozyme, we next asked whether M₂C competes with G(3′NH₂) for binding to the ribozyme, as inferred for G(2′NH₂). We therefore measured binding of G(3′NH₂) at various Mg²⁺ and Mn²⁺ concentrations (Figures S5 and S6). Surprisingly, increasing the concentration of Mg²⁺ from 2 to 100 mM weakened binding of G(3′NH₂) by only 4-fold (Figure S5), in contrast to the 60-fold decrease in the level of binding observed for G(2′NH₂). Furthermore, binding of G(3′NH₂) was unaffected by the addition of Mn²⁺ (Figure S5), whereas Mn²⁺ destabilizes G(2′NH₂) binding by at least a 100-fold. The observation that Mg²⁺ and Mn²⁺ have no or little effect on binding of G(3′NH₂) (Table S1) is consistent with a model in which this G analogue is bound in an alternative configuration, with the 3′-NH₂ positioned away from M₂C. Thus, although the adjacent G 2′- and 3′-OH groups both contact M₂C (Figure 1), replacing either −OH with an −NH₃⁺ modification leads to different outcomes in how these G analogues are bound within the active site.

To test the model described above and potentially learn more about these alternative binding modes, we determined the effects of removing the adjacent −OH group of G(2′NH₂) and G(3′NH₂) on binding to the ribozyme. If the interaction between M₂C and the G 2′-OH group of G(3′NH₂) (or the G 3′-OH group of G(2′NH₂)) were retained, abating the −OH moiety via −H substitution is, most simply, expected to destabilize binding. Alternatively, if these analogues can access alternative binding modes via the −NH₃⁺ substituent, the affinities of the analogues could remain the same or even increase. We therefore measured binding of protonated 3′-deoxy-2′-aminoguanosine and 2′-deoxy-3′-aminoguanosine [G(2′NH₃,3′H) and G(2′H,3′NH₃)], respectively (Table 1), following procedures analogous to those described above for G(3′NH₂) (see Figures S2 and S3).

The relative effects of −H and −NH₃⁺ substitutions on G binding to E-S are summarized in Figure 2. Whereas replacing the 2′- and 3′-OH groups with −H weakens binding of G, removal of the neighboring OH groups strengthens binding of G(2′NH₃) and G(3′NH₃), with G(2′NH₃,3′H) and G(2′H,3′NH₃) binding 10- and 470-fold stronger than G, respectively (Figure 2). In addition, Mg²⁺ and Mn²⁺ had little effect on binding of G(2′H,3′NH₃) (Table S2), suggesting that M₂C does not compete with this analogue for binding to the ribozyme. These results support binding in alternative modes within the active site of the Tetrahymena ribozyme, and the 1–3 kcal/mol strengthened binding presumably reflects new, fortuitous interactions made with the ammonium groups of G(2′NH₃) and G(3′NH₃) that are easier to access without steric restrictions to ribose ring motion and/or steric hindrance by the neighboring OH groups.

We considered two initial classes of models for the unexpected tight binding of G(2′H,3′NH₃) to E-S. (I) Major active site reorganization is required for G(2′H,3′NH₃) binding; this process would most simply be expected to slow binding by presenting an additional step and energetic barrier but decrease k_off because of the additional strong electrostatic interaction. (II) A strong electrostatic interaction tethers the G analogue as a first step in the binding process, increasing the binding rate by providing time for rearrangement to a binding competent active site conformation. As these models are not mutually exclusive, both factors could be in play. To evaluate these models, we probed k_on and k_off for G(2′H,3′NH₃).

To measure k_off for G(2′H,3′NH₃), we utilized a pulse-chase assay, which involves incubating the ribozyme with radiolabeled S and G(2′H,3′NH₃) at various times to form the E-S-G(2′H,3′NH₃) complex (Figure 3A). We subsequently diluted the sample 10-fold in a chase solution containing excess G to prevent rebinding of G(2′H,3′NH₃) that had dissociated from the ribozyme before or after addition.
of the chase. Under the conditions of our experiment, binding of G to E-S leads to rapid cleavage of S from the E-S-G complex with a rate constant of approximately 2 min⁻¹, providing us with a readout for monitoring changes in the fraction of E-S with G(2'-H,3'NH₃⁺) bound.

If the dissociation rate constant (k₉) for G(2'-H,3'NH₃⁺) is smaller than the rate of cleavage of S from the E-S-G complex (i.e., k₁₂ < 2 min⁻¹), cleavage of S following addition of the chase solution is expected to be biphasic, with a fast phase corresponding to the fraction of ribozyme without G(2'-H,3'NH₃⁺) bound and a slow phase corresponding to dissociation of G(2'-H,3'NH₃⁺) from the E-S-G(2'-H,3'NH₃⁺) complex that allows formation of the productive E-S-G complex. In contrast, if the G(2'-H,3'NH₃⁺) rate of dissociation exceeds that for cleavage of S from E-S-G (i.e., k₁₂ > 2 min⁻¹), cleavage of S is expected to be monophasic following addition of the chase, with a rate constant of 2 min⁻¹.

As shown in Figure 3B, we observed monophasic kinetics with a rate constant of ~2 min⁻¹ for cleavage of S in our pulse-chase experiments. This value, which is identical to the rate constant for cleavage of S from the E-S-G complex, indicates that k₁₂ is identical to the rate constant for cleavage of S from the E-S-G complex. This result is consistent with the idea that G(2'-H,3'NH₃⁺) binding is limited by the nucleation rate for forming 2 base pairs. 

**DISCUSSION**

RNA recognition of ligands is orders of magnitude slower than diffusion, indicating that most RNA/ligand collisions are not productive; i.e., they do not lead to formation of the stable bound complex. As this slow binding is observed for all natural and in vitro-selected RNAs studied to date, slow recognition may be a general property of RNA and thus a property that may have affected function in an RNA world, the transition to the modern-day protein world, and the ability to efficiently engineer RNA/ligand interaction and target RNA with drugs. Several potential mechanisms could be responsible for RNA’s slow binding and there is support from both structural and functional studies for the simplest of these models, required conformational rearrangements between the free RNA and the bound state that are unfavorable and slow (e.g., refs 61–64).

The fastest binding to RNA (other than by proteins, where RNA can be considered the ligand) is duplex formation (Figure 4). There, an initial unstable complex forms, presumably with a single base pair, that can either dissociate...
The residence time of E·G out is orders of magnitude below the rate of dissociation. The calculated equilibrium constant for formation of E·G out (\(k_{on}/k_{off} = 10^6 \text{ M}^{-1} \text{ min}^{-1}\)) is 10^4 \text{ M}^{-1}, suggesting that the initial binding step involves some weak stabilizing interactions. (C) Model and (D) free energy diagram for binding of UCGAAACC. Residues 5′ and 3′ to G (UC and AAACC, respectively) base pair with the ribozyme, forming the P9.0 and P10 helices, respectively (E·G out\(^{5′,10} \)). Rearrangement of the G binding site enables accommodation of G, forming E·G out\(^{P9.0/10} \). The free energy diagram for this process is shown in panel D (blue line), and for comparison, we show the profile for G from panel B (gray line). Prior data (ref 61) indicate the P9.0 and P10 helices increase the residence time of E·G out\(^{5′,10} \) such that the rate of association for UCGAAACC (\(k_{on} \approx 10^6 \text{ M}^{-1} \text{ min}^{-1}\), similar to rate constants for duplex formation. From a \(k_{accom} \approx 10^4 \text{ min}^{-1}\), the equilibrium constant for formation of E·G out\(^{P9.0/10} \) (\(K_{eq}^{P9.0/10}\)) is calculated to be 10^4 \text{ M}^{-1} \cdot (10^6 \text{ M}^{-1} \cdot \text{min}^{-1})/(10^4 \text{ M}^{-1} \cdot \text{min}^{-1})\). (E) Model and (F) free energy diagram for binding of G(2′H,3′NH\(_3\))\(^+\). The positively charged amino group of G(2′H,3′NH\(_3\))\(^+\) (colored green; the 2′-H substituent is not shown for the sake of simplicity) forms favorable interactions with the ribozyme (denoted by hatched lines) within E·G out\(^{NH3}^+\). This “binding anchor” increases the residence time of E·G out\(^{NH3}^+\) for subsequent accommodation of the G analogue. The interaction made with the NH\(_3\)\(^+\) substituent in E·G out\(^{NH3}^+\) may or may not be retained within E·G out\(^{NH3}^+\), and this is denoted by a question mark in panel E. The free energy diagram for this process is shown in panel F (green line), and for comparison, we show the profile for G from panel B (gray line). The data from Table 3 suggest that the –NH\(_3\)\(^+\) substituent on G(2′H,3′NH\(_3\))\(^+\) stabilizes E·G out\(^{NH3}^+\) so that \(k_{on} \approx 10^7 \text{ M}^{-1} \text{ min}^{-1}\). From a \(k_{accom} \approx 10^4 \text{ min}^{-1}\), the equilibrium constant for formation of E·G out\(^{NH3}^+\) (\(K_{eq}^{NH3}^+\)) is calculated to be = 10^7 \text{ M}^{-1} (=k_{on}/k_{accom}).

(nonproductive binding) or allow formation of a second and then third base pair, etc. to give stable complex formation. While dissociation of the single-base pair complex is more likely than formation of the next pair, its hydrogen bonds cause it to persist longer than a simple encounter complex that lacks stabilizing interactions, thereby making formation of the second interaction (in this case another base pair) more likely than it would otherwise be.

Thus, interactions that can increase the lifetime of an early binding complex can increase the efficiency and rate of binding. Such a mechanism was found for guanosine (G) recognition by the Tetrahymena group I intron and may play a role in the specificity of self-splicing \(^{41}\) (Figure 5A,B). Pre-steady-state kinetic studies revealed a “gating” step in G binding to the Tetrahymena ribozyme with an estimated rate constant of \(\sim 10^4 \text{ min}^{-1}\), such that most G molecules dissociate instead of stabilizing interactions, thus leading to formation of a weak complex between G and the ribozyme (E·G out\(^{5′,10} \)). The G binding site on the ribozyme must then undergo a conformational rearrangement to accommodate G (E·G out\(^{P9.0/10} \)), allowing formation of a second and/or 3′ to the G that can form base pairs (Figure 4) adjacent to the G site.

Figure 5. Free energy diagrams for effects of binding substeps on association and dissociation kinetics for G and G analogues with the Tetrahymena ribozyme. (A) Model and (B) free energy diagram for binding of G. A diffusion-limited step leads to formation of a weak complex between G and the ribozyme (E·G out\(^{5′,10} \)). The G binding site on the ribozyme must then undergo a conformational rearrangement to accommodate G (E·G out\(^{P9.0/10} \)) to form E·G in. Prior data (ref 61) indicate that the rate of this step is slow (\(k_{accom} \approx 10^4 \text{ min}^{-1}\)) such that most G molecules dissociate instead of binding to the G binding site, and the observed association rate constant for G (\(k_{on} \approx 10^5 \text{ M}^{-1} \text{ min}^{-1}\)) is orders of magnitude below the rate of diffusion. The calculated equilibrium constant for formation of E·G out\(^{P9.0/10} \) (\(K_{eq} \approx k_{on}/k_{accom} = (10^5 \text{ M}^{-1} \cdot \text{min}^{-1})/(10^4 \text{ M}^{-1} \cdot \text{min}^{-1})\)) is 10^4 \text{ M}^{-1}, suggesting that the initial binding step involves some weak stabilizing interactions. (C) Model and (D) free energy diagram for binding of UCGAAACC. Residues 5′ and 3′ to G (UC and AAACC, respectively) base pair with the ribozyme, forming the P9.0 and P10 helices, respectively (E·G out\(^{5′,10} \)). Rearrangement of the G binding site enables accommodation of G, forming E·G out\(^{P9.0/10} \). The free energy diagram for this process is shown in panel D (blue line), and for comparison, we show the profile for G from panel B (gray line). Prior data (ref 61) indicate the P9.0 and P10 helices increase the residence time of E·G out\(^{5′,10} \) such that the rate of association for UCGAAACC (\(k_{on} \approx 10^6 \text{ M}^{-1} \text{ min}^{-1}\), similar to rate constants for duplex formation. From a \(k_{accom} \approx 10^4 \text{ min}^{-1}\), the equilibrium constant for formation of E·G out\(^{P9.0/10} \) (\(K_{eq}^{P9.0/10}\)) is calculated to be 10^4 \text{ M}^{-1} \cdot (10^6 \text{ M}^{-1} \cdot \text{min}^{-1})/(10^4 \text{ M}^{-1} \cdot \text{min}^{-1})\). (E) Model and (F) free energy diagram for binding of G(2′H,3′NH\(_3\))\(^+\). The positively charged amino group of G(2′H,3′NH\(_3\))\(^+\) (colored green; the 2′-H substituent is not shown for the sake of simplicity) forms favorable interactions with the ribozyme (denoted by hatched lines) within E·G out\(^{NH3}^+\). This “binding anchor” increases the residence time of E·G out\(^{NH3}^+\) for subsequent accommodation of the G analogue. The interaction made with the NH\(_3\)\(^+\) substituent in E·G out\(^{NH3}^+\) may or may not be retained within E·G out\(^{NH3}^+\), and this is denoted by a question mark in panel E. The free energy diagram for this process is shown in panel F (green line), and for comparison, we show the profile for G from panel B (gray line). The data from Table 3 suggest that the –NH\(_3\)\(^+\) substituent on G(2′H,3′NH\(_3\))\(^+\) stabilizes E·G out\(^{NH3}^+\) so that \(k_{on} \approx 10^7 \text{ M}^{-1} \text{ min}^{-1}\). From a \(k_{accom} \approx 10^4 \text{ min}^{-1}\), the equilibrium constant for formation of E·G out\(^{NH3}^+\) (\(K_{eq}^{NH3}^+\)) is calculated to be 10^7 \text{ M}^{-1} (=k_{on}/k_{accom}).

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results in longer residence times and more efficient (faster) binding (Figure 5C,D). When these residence times become longer than the gating time (i.e., longer than \( \sim 1/10^4 \text{min}^{-1} \) or \( \sim 10 \text{ms} \)), then G, in its oligonucleotide form, can bind as fast as the upstream or downstream helix forms, with a rate constant of \( \sim 10^8 \text{M}^{-1} \text{min}^{-1} \) as is typical for RNA duplex formation (Figure 5C,D).

Our observed faster binding of a G analogue with a positively charged amino group appears to be another manifestation of this binding mechanism (Figure 5E,F). According to this model, the positively charged amino group of G(2′H,3′NH₃⁺) provides a “binding anchor” that increases the residency time of an early complex in the binding and thus association rate constants. Indeed, the observation of an increase in the association rate constant, relative to that of G, indicates that the –NH₃⁺ interaction is formed prior to the transition state for complex formation.

As RNAs are replete with negatively charged phosphoryl groups and other hydrogen bond acceptors, the introduction of well-placed –NH₃⁺ groups or other positively charged moieties may provide a generalizable means for enhancing binding rates. Our data indicate an association rate enhancement effect of at least 50-fold [\( \sim 10^8 \text{M}^{-1} \text{min}^{-1} \)] relative to that of G, and an observed faster binding of a G analogue with a positively charged amino group appears to be fortuitous, considerably larger effects may be possible. Such anchors could even be transient, holding binding groups near their binding sites until rearrangements allow for additional interactions, with subsequent rearrangement to a more stable final bound state that does not contain the –NH₃⁺ interaction. (In this case, the –NH₃⁺ group would catalyze formation and release of the ligand.)

We speculate than an anchor mechanism may contribute to the efficacy of ribosome binding aminoglycoside antibiotics. These small molecules have been shown to bind to numerous RNAs, likely at multiple sites, so their enhanced by fast binding that allows binding to and trapping of a transient ribosomal state. With the renewed interest in drugs for targeting RNAs, it is important to continue to develop our fundamental understanding of the kinetics and thermodynamics of RNA/ligand interactions.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.9b00231.

- pH dependence of binding of G and G analogues to the ribozyme (PDF)

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**ADDITIONAL NOTES**

“The pulse–chase assay provides valuable information for binding of G(2′H,3′NH₃⁺), but the kinetic parameters obtained are limits and were obtained indirectly. To obtain values for these rate constants and to probe complex binding mechanisms that may involve more than two observable states (e.g., Figure 5), 2-aminopurine riboside and its corresponding derivatives with –NH₂⁻ and/or –H substituents might be used with mutant ribozymes to follow binding in real time via 2-aminopurine fluorescence (e.g., refs 73 and 74).

“The effect of the –NH₃⁺ substituent on the binding of G to the ribozyme presumably arises from electrostatic interactions (see Figures S1–S6). However, additional contributors, including differences in hydrogen bonding, steric accessibility, sugar pucker, and inductive effects among the G analogues, are possible and remain to be tested (e.g., ref 75).

**REFERENCES**


