Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 8362–8366, September 1993
Biochemistry

Guanosine binding to the *Tetrahymena* ribozyme: Thermodynamic coupling with oligonucleotide binding

(RNA catalysis/equilibrium dissociation constants/kinetics/cooperativity)

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ABSTRACT **The L-21 *Sca* I ribozyme derived from the group I intron of *Tetrahymena thermophila* pre-rRNA catalyzes an endonuclease reaction analogous to the first step of self-splicing. Guanosine (G) is bound by the ribozyme, and its 3′-hydroxyl group acts as the nucleophile. Here, we provide evidence that K_m for G in several single-turnover reactions is equal to the equilibrium dissociation constant for G. This evidence includes the observation that removal of the 2′-hydroxyl group at the cleavage site of the oligoribonucleotide substrate [from CCCUCUA to CCCUC(dU)A] decreases the rate of cleavage ≈1000-fold but has no effect on either the K_m for G (0.17 mM) or for guanosine 5′-monophosphate (pG) (0.09 mM). In the course of this study, it was observed that K_m for G or pG was lower by a factor of 5 for reactions with the ribozyme–CCCUC(dU)A complex compared with the free ribozyme, indicating a modest amount of thermodynamic coupled binding of the two substrates. The decrease in the rate of oligonucleotide dissociation upon addition of saturating pG provides independent support for this coupling. Coupling is lost with a substrate that cannot make the normal tertiary interactions with the ribozyme, providing evidence that coupled binding requires docking of the substrate into the catalytic core. Surprisingly, the binding of product CCCUCU and G is slightly anticooperative, indicating that the cleaved pA is important for coupling with substrate. Coupled binding suggests a splicing model in which the intron binds G tightly to promote the first step of the reaction, after which its binding is an order of magnitude weaker, thereby facilitating the second step.**

The 413-base intron of *Tetrahymena thermophila* nuclear pre-rRNA splices in the absence of protein (1, 2). In the first of two transphosphoesterification steps, exogenous guanosine (G) or one of its 5′ phosphorylated forms binds to the intron and attacks at the 5′ splice site. The products of the first step are the 5′ exon ending with a 3′-hydroxyl group and the intron–3′ exon species with the attacking G covalently linked at the 5′ end. These products then undergo the second step of splicing, a reaction that is chemically equivalent to the reverse of the first step with the 3′-terminal guanosine of the intron at position 414 (G414) substituting for the exogenous G. This reaction produces ligated exons and an excised intron.

The L-21 *Sca* I RNA is a shortened form of the intron that catalyzes an intermolecular reaction analogous to the first step of splicing (Fig. 1). This form of the intron cleaves an RNA substrate of specific sequence with multiple turnover and, thus, can be considered an RNA enzyme, or "ribozyme." Kinetic and thermodynamic characterization of this ribozyme has led to a new level of mechanistic understanding of the intron chemistry and biology (10).

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In previous work, however, assumptions were made in determining the ribozyme's affinity for G (11–13). These assumptions are tested herein. In the course of these experiments, we observed coupled binding of G and oligonucleotide, indicating structural and functional communication between the substrates in this active site composed of RNA. Additionally, these findings suggest a model in which the intron manipulates the binding of G to promote self-splicing.

METHODS

L-21 *Sca* I Ribozyme. Ribozyme was prepared by transcription of *Sca* I-cut pT7L-21 DNA template using phage T7 RNA polymerase with purification by gel electrophoresis and size-exclusion chromatography (14). Ribozyme concentration was determined spectrophotometrically (14).

Oligonucleotide Substrates and Products. Oligonucleotides were synthesized chemically on an Applied Biosystems model 380B DNA synthesizer as described (15, 16) by using phosphoramidites [Applied Biosystems, American Bionetics (Hayward, CA), or Milligen Biosystems (Novato, CA)]. Oligonucleotides were 5′-end-labeled by treatment with phage T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (New England Nuclear) and were purified as described (4). Concentrations were determined based on specific activity.

Kinetics. Single-turnover reactions were performed with <5 nM of labeled oligonucleotides and excess (≥10-fold) ribozyme. Reactions were carried out at 50°C or 30°C in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes), sodium salt, pH 7.0 or pH 5.5 (pH values determined at 25°C). Ribozyme was preincubated at 50°C in the presence of buffer and Mg^{2+} for 20 min to allow formation of a single active folded species (≥90%) of ribozyme (ref. 12; P. Legault, T.S.M., and D.H., unpublished results). For reactions at 30°C, the samples were subsequently preincubated at 30°C for 5 min. Reactions were initiated by simultaneous addition of labeled oligonucleotide and G or guanosine 5′-monophosphate (pG). Typically, six aliquots (≈2 μl each) were removed from a reaction mixture (20 or 40 μl) and quenched with 2–3 volumes of stop buffer containing 80% formamide, 50 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol, and 2 mM Tris borate, pH 7.5. Reaction products were separated on 20% polyacrylamide/7 M urea gels. When reactions were followed for more than 2 hr, the mixtures were kept submerged and/or were centrifuged periodically to prevent concentration of the sample by evaporation. The fraction of substrate remaining relative to total substrate and

Abbreviations: pG, guanosine 5′-monophosphate; E, the *Tetrahymena* ribozyme; S, an oligonucleotide substrate whose identity depends on the experiment; k_c, the rate constant for the chemical step, which is equal to k_{cat} for the single-turnover reactions herein.

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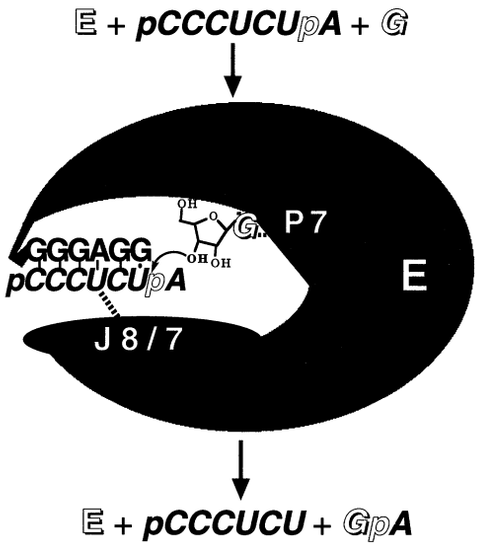

FIG. 1. The ribozyme endonuclease reaction with the all RNA substrate, CCCUCUA: 5′-CCCUCUA + G → 5′-CCCUCU + GA. Substrate binds to the internal guide sequence, 5′-GGAGGG, of the ribozyme, forming Watson–Crick base pairs except for the phylogenetically conserved G·U wobble pair at the cleavage site. Previous studies have provided evidence for tertiary interactions between the 2′-hydroxyl of U(−3) of the substrate and the J8/7 region (nucleotides 299–306) of the ribozyme, represented here by a dashed line (3). The extra binding energy from these interactions allows the substrate to be more tightly bound than predicted for a simple duplex. There is evidence for a two-step binding model that involves first base pairing (open complex) and then formation of tertiary interactions (closed complex) (4, 5). The second substrate of this reaction is G, or a guanine nucleotide, which binds to the P7 helix (6–9).

product at each time point was quantitated with a PhosphorImager (Molecular Dynamics).

Determining K_m(G) Values. Except where mentioned, first-order kinetics were observed for ≈3 half-lives with end points of 95% reacted at 50°C and 90% at 30°C, indicating a single species (≥90%) of oligonucleotide substrate. Values for the Michaelis–Menten constant for G, K_m(G), were then obtained from a nonlinear least-squares fit (KaleidaGraph, Synergy Software, Reading, PA) to the dependence of the observed rate constant on the G molar concentration ([G]). For reactions with ribozyme saturating with respect to oligonucleotide binding, the equation $k_{obs} = k_c\,[G]/\{K_m(G) + [G]\}$ was used, where k_c is the rate constant for cleavage of the oligonucleotide substrate (S) with saturating G (i.e., reaction of E·S·G, where E is the *Tetrahymena* ribozyme and S is an oligonucleotide substrate whose identity depends upon the experiment). For reactions with ribozyme subsaturating with respect to oligonucleotide binding, the equation $k_{obs} = (k_{cat}/K_m)^S\,[E][G]/\{K_m(G) + [G]\}$ was used, where the value $(k_{cat}/K_m)^S$ is the second-order rate constant with saturating G (i.e., reaction of E·G + S), and [E] is the molar concentration of E. Cleavage of oligonucleotides in the absence of G (12) accounted for <2% of the maximal observed rate. This background rate was subtracted from each data point, although this had no effect on the fit. All data fit these equations well, and the errors reported contain the range of values obtained from independent experiments. At least two independent experiments were performed for each curve, and direct comparisons were made from experiments performed side-by-side.

For each determination of K_m(G), the [E] was varied at both the lowest and highest [G] or [pG]. The observed rate constant was shown to increase linearly, within 15%, over a range ≥ 4-fold of [E] for reactions using subsaturating ribozyme (E·G + S). In contrast, the observed rate was shown to be independent, within 10%, over a range ≥ 4-fold of [E] for reactions using saturating ribozyme (E·S·G). Thus, over the range of [G] or [pG], there is no change in molecularity of the reaction with respect to ribozyme and oligonucleotide; this demonstrates that the equations above used to fit the data are valid over the concentration range.

Because of the tendency of G to aggregate or precipitate at high concentrations, the concentration of free G was determined. A mutant ribozyme (A264:U311) that significantly weakens the binding of the G cofactor (7, 8) was used to determine the concentration of active G by measuring the second-order rate constant $(k_{cat}/K_m)^G$. Based on these measurements, we restricted our experiments to [G] ≤ 4 mM at 50°C, [G] ≤ 2 mM at 30°C, and [pG] ≤ 8 mM at 30°C.

Pulse–Chase Experiments. In all cases, ribozyme was preincubated in 10 mM Mg^{2+}/50 mM Mes, pH 5.5, as described above. To measure the effect of pG on the rate constant of oligonucleotide association, labeled CCCUC(dU)A with or without 2 mM pG was added to 5–15 nM ribozyme to initiate binding. The low pH minimized the endonuclease reaction during this time. At various times t_1 after initiation, aliquots were diluted 1:9 fold into a chase solution containing excess unlabeled CCCUCU (1–4 μM, which is >60[E]), 1.5 mM G, and 50 mM *N*-(2-hydroxyethyl)piperazine-N′-3-propanesulfonic acid (EPPS) (pH 8.8) to prevent further binding of CCCUC(dU)A while allowing bound CCCUC(dU)A to react. An aliquot from each chase solution was quenched in stop buffer after 10 min ($\gg t_{1/2}$ = 1.5 min for the reaction in the chase). The value for the association rate constant (k_1) was obtained from the observed rate constant, where $k_1 = (k_{obs} - k_{-1})/[E]$ and k_{-1} is the rate constant for dissociation. To measure the rate of oligonucleotide dissociation, 0.2–1 μM E was allowed to completely bind labeled CCCUC(dU)A during a set time, t_1 = 1 min, after initiation. Then the sample was diluted 1:1 in chase solution containing unlabeled CCCUCU (20–40 μM, which is ≥20[E]), 10 mM Mg^{2+}, and 50 mM Mes (pH 5.5) with or without 2 mM pG. At various times t_2, aliquots were removed and further diluted 1:9 in excess unlabeled CCCUCU (1–4 μM, which is >20[E]), 1.5 mM G, and 50 mM EPPS (pH 8.8), from which an aliquot was quenched in stop buffer after 10 min. The dissociation rate constant (k_{-1}) was determined by using the equation: $k_{-1} = k_{obs} - k_c \cong k_{obs}$, because $k_c = 6.6 \times 10^{-4}$ min^{-1}. Changing the order of addition of the pH 5.5 chase solution and the labeled oligonucleotide resulted in negligible reaction (≈1% over 3 hr), demonstrating that the chase was effective. Varying the ribozyme and oligonucleotide concentrations did not affect the observed results of either experiment, except that the rate of substrate association depended linearly on [E] as predicted (12).

RESULTS AND DISCUSSION

Evidence That K_m(G) Equals the Dissociation Constant for G. It has been argued that the value of K_m(G) does not equal K_d(G) for several reactions of the ribozyme (see *Implications*). In general, K_m cannot be assumed to equal K_d (17), as shown in the following three examples.

(*i*) In the simple reaction scheme of Eq. **1**, K_m(G) equals $(k_{-1} + k_c)/k_1$. When $k_{-1} \gg k_c$, then K_m(G) equals k_{-1}/k_1, which by definition is K_d(G). If k_c is not much less than k_{-1}, then K_m(G) will be greater than K_d(G).

$$E{\cdot}S + G \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}S{\cdot}G \xrightarrow{k_c} \text{products} \qquad [1]$$

(*ii*) If there is any intermediate that builds up subsequent to the formation of E·S·G, then K_m(G) will be less than the true K_d(G).

(*iii*) If there is a change in the rate-limiting step to a step other than those shown in Eq. **1** as [G] is changed, then K_m(G)

will be less than K_d(G). This occurs because the rate levels off prior to G being fully bound to the ribozyme.

The current investigation exclusively utilizes single-turnover reactions because they involve fewer steps than multiple-turnover reactions, minimizing such complications as *ii* and *iii* above.

If the binding of G were not in rapid equilibrium prior to the cleavage step for a single-turnover reaction, then a steady-state concentration of bound G species would never be established. This condition would result in an initial lag in the reaction rate, because product formation could not begin until after binding. The resulting inability to assign a rate constant to the reaction at each [G] value would prevent $K_m(G)$ from being determined. Such a lag would be most obvious when $k_1[G] \simeq k_c$. No lag was observed (data not shown), suggesting that the G binding equilibrium is established rapidly. This observation suggests that K_m(G) for a single turnover reaction could equal K_d(G).

To further test the assumption that K_m(G) is equal to K_d(G), the rate constant of chemical cleavage (k_c in Eq. 1) was changed. Changing the nucleotide at the cleavage site from ribose to deoxyribose reduces k_c by ≈3 orders of magnitude, while decreasing oligonucleotide binding only by a factor of ≈3 (18). If k_c contributed significantly to K_m, then a change in k_c of ≈3 orders of magnitude would have a profound effect on K_m(G). A priori, one cannot predict what effect the removal of a functional group that drastically affects the chemical step will have on the binding of G. However, the data in Fig. 2 show that K_m(G) was unaffected by this 2′-hydroxyl group. Similar results were obtained for pG reacting with the same ribozyme–substrate complexes [K_m(pG) = 0.09 ± 0.01 mM and k_c = 7.6 ± 0.6 × 10^{-4} min^{-1} for E·CCCUC(dU)A; K_m(pG) = 0.09 ± 0.01 mM and k_c = 0.8 ± 0.04 min^{-1} for E·CCCUCUA; pH 5.5, 30°C]. Because the change in k_c does not affect K_m(G), there is no evidence for a change to a different rate-limiting step (condition *iii*) or for the value for k_{-1} being significantly smaller than k_c (condition *i*).

An independent approach to vary the maximal rate and determine its effect on K_m is to vary the pH. The values of K_m(G) of 0.8 ± 0.3 mM and 1.1 ± 0.2 mM at pH 5.5 and 7.0, respectively, for a reaction with subsaturating ribozyme are the same despite the ≈50 fold difference in maximal rate [for S· = d(CCCUC)Ud(AAAAA), $(k_{cat}/K_m)^S$ = 3.4 ± 0.8 × 10^4 M^{-1}·min^{-1} at pH 5.5 (data not shown) and 2.3 × 10^6 M^{-1}·min^{-1} at pH 7.0 (Fig. 3); 50°C]. With ribozyme saturating with respect to CCCUC(dU)A, K_m(G) is also independent of pH, with values of 0.15 ± 0.03 mM (data not shown) and 0.17

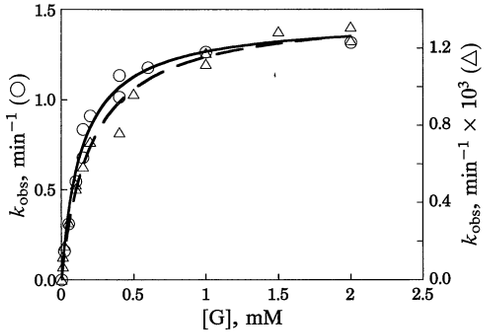

FIG. 2. A large change in the rate of the chemical step has no effect on K_m(G). The rates of cleavage of CCCUCUA (○) and of CCCUC(dU)A (△), each bound to 400 nM ribozyme, were determined as a function of [G] at pH 5.5 and 30°C. The cleavage of CCCUC(dU)A was measured by initial rates, because the rate was slow. The lines represent fits to the data with K_m = 0.19 ± 0.03 mM and k_c = 1.4 ± 0.1 min^{-1} for E·CCCUCUA (—), and K_m = 0.16 ± 0.03 mM and k_c = 1.4 ± 0.1 × 10^{-3} min^{-1} for E·CCCUC(dU)A (---).

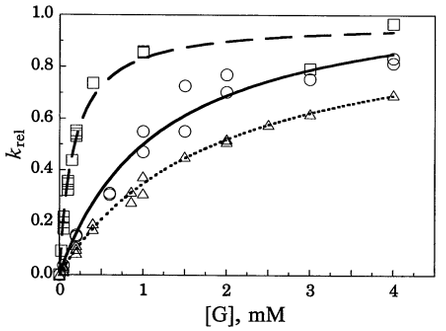

FIG. 3. G binding is stabilized by bound oligonucleotide substrate and destabilized by bound product. The dependence on [G] of the observed reaction rates of CCCUC(dU)A (□) with saturating ribozyme (500 nM), of d(CCCUC)Ud(AAAAA) (○) with subsaturating ribozyme (200 nM), and of CCCUCU (△) with saturating ribozyme (100 nM) was measured at pH 7.0 and 50°C. The cleavage of CCCUCU was measured by initial rates because the rate was slow. The lines represent fits to the data with K_m = 0.17 ± 0.03 mM and k_c = 0.55 ± 0.04 min^{-1} for E·CCCUC(dU)A (dashed line); K_m = 1.1 ± 0.2 mM and $(k_{cat}/K_m)^S$ = 2.3 ± 0.3 × 10^6 M^{-1}·min^{-1} for E + d(CCCUC)Ud(AAAAA) (solid line); and K_m = 1.8 ± 0.3 mM and k_c = 1.2 ± 0.1 × 10^{-2} min^{-1} for E·CCCUCU (dotted line). The data have been normalized such that k_{rel} = 1 is the maximal rate constant for each curve.

mM (Fig. 3) at pH 5.5 and 7.0, respectively [k_c = 0.015 min^{-1} and 0.55 min^{-1}, respectively; 50°C].

Together, these approaches provide evidence that K_m equals K_d in these reactions. Furthermore, the K_m(G) values differ depending on the identity of the oligonucleotide and depending on whether the ribozyme is saturated with the oligonucleotide. These differences suggest thermodynamic coupling between G and oligonucleotide binding.

G Binds More Strongly to the E·CCCUC(dU)A Complex Than to the Free Ribozyme. Fig. 3 shows that G binding to E·CCCUC(dU)A [K_m(G) = 0.17 ± 0.03 mM] is ≈6 fold stronger than to the free ribozyme [K_m(G) = 1.1 ± 0.2 mM]. Since the rate of the chemical cleavage step for E·G·CCCUCUA is fast [calculated to be ≈350 min^{-1} (12)], the use of an oligonucleotide substituted with a deoxyuridine at the cleavage site allows an accurate measurement of the rate constant for the chemical step (k_c = 0.55 min^{-1}). The value of K_m(G) for the reaction with free ribozyme was obtained under subsaturating conditions [$(k_{cat}/K_m)^S$] for the substrate d(CCCUC)Ud(AAAAA), which binds weakly (K_d ≥ 5 μM; D.H., unpublished results). Use of a weak-binding oligonucleotide is necessary because its fast dissociation rate prevents the rate of oligonucleotide association from becoming limiting over the [G] range (condition *iii*), a situation encountered with a tighter binding oligonucleotide (11).

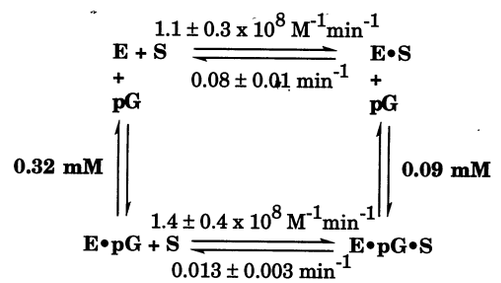

FIG. 4. Coupled binding is revealed in the oligonucleotide dissociation rate constant. This scheme represents the energetics between pG and CCCUC(dU)A on the ribozyme at pH 5.5 and 30°C. The binding constants for pG determined in Fig. 5 are shown as well as the individual rate constants obtained from pulse–chase experiments performed at pH 5.5 and 30°C.

Bound G Slows the Rate of Oligonucleotide Dissociation. If the presence of an oligonucleotide bound to the ribozyme affects G affinity, then bound G must affect the binding of the oligonucleotide. Pulse–chase experiments measuring individual rate constants were used to test this prediction. The results summarized in Fig. 4 show that there is a decrease by a factor of 5 in the dissociation rate constant of CCCUC(dU)A with pG bound to the ribozyme; however, the association rate constant of CCCUC(dU)A is unchanged by the presence of pG. These data provide independent confirmation of the coupled binding (Figs. 3 and 5) and of the conclusion that K_m(G) equals K_d(G) for the measurements herein.

G Binds More Weakly to the E·CCCUCU Complex. Fig. 3 shows the dependence on [G] of a secondary reaction, cleavage of CCCUCU to CCCU (Eq. 2) in the presence of saturating ribozyme (4).

$$\text{E·CCCUCU} + \text{G} \overset{K_d(\text{G})}{\rightleftharpoons} \text{E·G·CCCUCU} \rightleftharpoons$$

$$(\text{E·G·CCCUCU})'' \xrightarrow{k_c''} \text{E·GCU·CCCU} \qquad [2]$$

The E·CCCUCU complex is analogous to the state of the intron that exists after the first step of splicing, and (E·G·CCCUCU)'' has the RNA duplex containing the bound oligonucleotide translocated from its normal position, so that cleavage by G occurs two nucleotides preceding the normal site of reaction (4). The K_m(G) = 1.8 mM is larger than values obtained for reactions with the free ribozyme or with the E·CCCUC(dU)A complex. Like E·CCCUC(dU)A, E·CCCUCU is most stable as a closed complex (see Fig. 1 and *Implications*) as has been argued previously (4). G binds to this form, as there is evidence that all intermediates [including (E·G·CCCUCU)''] between E·G·CCCUCU and the chemical step (k_c'', Eq. 2) are in equilibrium and account for a small fraction of the ribozyme species in the reaction (see condition *ii* above; ref. 4). Thus, in the equilibrium equation it is E·G·CCCUCU that predominates as the observed bound species, even though cleavage occurs from (E·G·CCCUCU)''. In a similar experiment, bound product with a single deoxyribonucleotide at the −1 position, CCCUCdU, was cleaved to CCCU; K_m(G) = 2.2 ± 0.6 mM is the same within error as that for CCCUCU (data not shown).

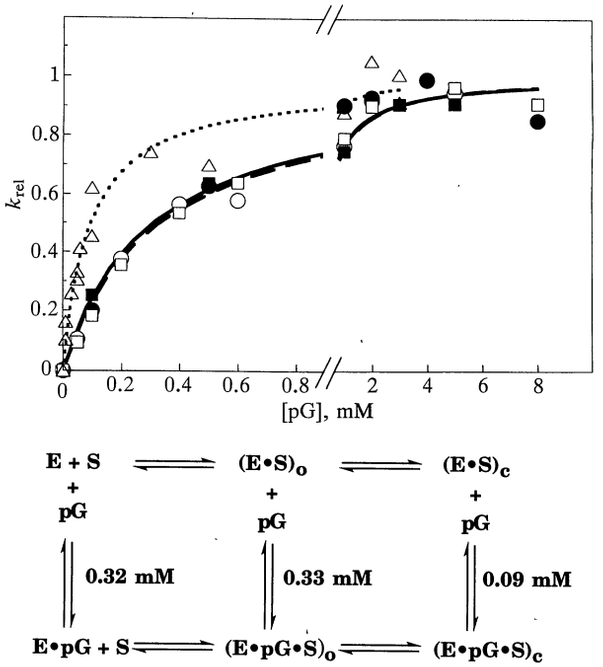

FIG. 5. (*Upper*) Coupled binding is substrate specific. The dependence on [pG] of the reaction rates of CCCUCUA with saturating ribozyme (500 nM) (△) and of d(CCCUC)Ud(AAAAA) with saturating (2000 nM) (○,●) and subsaturating ribozyme (20 nM) (□,■) was measured at pH 5.5 and 30°C. Open and closed symbols represent independent experiments which were normalized to their respective rates at 8 mM pG because ribozyme activity can vary as much as 2-fold in independent experiments. For visual comparison, all data were subsequently normalized such that k_{rel} = 1 is the maximal rate constant for each curve. The lines represent fits to the data with K_m = 0.09 ± 0.01 mM and k_c = 0.80 ± 0.04 min^{-1} for E·CCCUCUA (····); K_m = 0.33 ± 0.03 mM and k_c = 1.1 ± 0.1 × 10^{-2} min^{-1} for E·d(CCCUC)Ud(AAAAA) (– – –); and K_m = 0.32 ± 0.04 mM and $(k_{cat}/K_m)^S$ = 6.0 ± 0.8 × 10^4 M^{-1}·min^{-1} for E + d(CCCUC)Ud(AAAAA) (—). (*Lower*) The relationship between the binding affinities for pG (*Upper*) and the ribozyme states to which pG is binding. Analogous results were obtained with G (data not shown). Subscripts o and c represent the open and closed complexes, respectively (Fig. 1).

The dissociation rate constant of CCCUCU in the absence of G from pulse–chase experiments is less by a factor of 2–3 than that obtained with saturating G from multiple-turnover experiments (data not shown). Furthermore, with increasing G there is an increase in the rate of multiple-turnover, a reaction in which the dissociation rate constant of CCCUCU is predominantly rate-limiting (data not shown). Thus, in contrast to the decrease in the dissociation rate constant of CCCUC(dU)A (Fig. 4), bound G increases the dissociation of CCCUCU. This is expected from the results in Fig. 3, which show binding of G being destabilized in the presence of saturating CCCUCU. This destabilization is consistent with data in the accompanying paper (19).

Bound d(CCCUC)Ud(AAAAA) Does Not Enhance Binding of pG. Binding of this oligonucleotide cannot involve the tertiary interactions depicted in Fig. 1 because these involve 2′-hydroxyl groups that are missing in the predominantly deoxyribose substrate (3). As depicted in Fig. 5 *Upper*, the binding of pG to E·d(CCCUC)Ud(AAAAA) and to free ribozyme are nearly identical [K_m(pG) = 0.33 mM and 0.32 mM, respectively]. However, the binding of pG is ≈4-fold stronger to E·CCCUC(dU)A [K_m(pG) = 0.09 mM]. The weaker pG affinity of the free ribozyme relative to E·CCCUC(dU)A is similar to that for G at 50°C and pH 7.0 (Fig. 3). Also, Figs. 2 and 5 show that, although pG binds more tightly than G by about a factor of 2, the maximal rate, k_c, is slowed by a factor of two, suggesting that the slightly tighter binding is nonproductive.

Implications. Previous determinations suggested that G and oligonucleotide binding are independent, although it was stated that a modest amount of coupling would have gone undetected (11, 12). The previous values estimated for K_d(G) are consistent with those presented here; however, those measurements did not have sufficient precision to detect the ≈5-fold coupled binding observed here. For DNA cleavage, the K_m(G) of 1 mM has been measured (20) and argued to equal K_d(G) (11, 13). This value is consistent with the K_d(G) to the free ribozyme presented here and with that determined by equilibrium dialysis (21). However, it has also been suggested that the values of K_m(G) in several single- and multiple-turnover reactions of the ribozyme do not represent K_d(G) (7, 11). This is because over the range of [G] used in these studies, there were changes in the rate-limiting step from cleavage to oligonucleotide association and dissociation for the single- and multiple-turnover experiments, respectively. Now, having measurements for the binding of G allows calculation of the energetics of the ribozyme's interaction with G: K_d(G) = 0.17 ± 0.03 mM corresponds to $\Delta G°$ = −5.5 ± 0.2 kcal/mol for binding of G to the E·S complex at 50°C.

The binding of G appears independent of both pH (above) and temperature [K_m(G) = 0.15 ± 0.03 mM (data not shown) and 0.16 mM (Fig. 2) at 50°C and 30°C, respectively, for

E·CCCUC(dU)A at pH 5.5]. On the basis of current models (8, 9), one would not expect that any functional group would change its protonation state to bind G, but it is unexpected that guanosine binding does not change over a 20-degree temperature range.

The closer examination of G binding by the ribozyme has revealed thermodynamic coupling between the binding of pG or G and CCCUC(dU)A (Figs. 3–5). Coupled binding of pG (or G) occurs in the closed complex that exists before chemical cleavage (Fig. 5 *Lower*). The closed complex is a ribozyme–oligonucleotide species that has gained binding energy from tertiary interactions involving the oligonucleotide's 2′-hydroxyl groups and the ribozyme's core (see Fig. 1). In contrast, the open complex appears to require only the Watson–Crick base pairing between the oligonucleotide and the ribozyme (4, 5). The substrate d(CCCUC)Ud(AAAAA) binds mainly in the open complex (D.H., unpublished results), and there is no coupling of binding with this substrate (Fig. 5). Thus, the evidence presented supports the model that the open and closed complexes are structurally distinct. In addition, this work demonstrates an energetic interaction between two substrates for an RNA catalyst, a phenomenon that appears common among proteins.

The weaker binding of G to E·CCCUCU (Fig. 3) indicates that the reactive phosphate or the 3′ A is important for the interaction between G and the oligonucleotide substrate. The reactive phosphate binds close to the G nucleophile for cleavage to occur and may provide this modest contribution (≈1 kcal/mol) to the ground-state binding of G. The two substrates might interact directly, or the interaction might be mediated by a component of the ribozyme structure, metal ion, or solvent. The ability of a single mutation to affect both oligonucleotide and G binding (22) may provide precedent for an indirect effect.

The ≈10-fold stronger binding of G to E·CCCUC(dU)A than to E·CCCUCU suggests that the intron modulates the binding of G through the splicing reaction. The stronger binding of G to E·CCCUC(dU)A leads to the proposal that the intron can bind G tightly to facilitate the first step of splicing. After the first step, a decrease in affinity for exogenous G would reduce competition for binding of the 3′-splice-site guanosine (G414) into the guanosine-binding site, leading to the second step of splicing. Such destabilization is suggested by the weaker binding of G to E·CCCUCU. For such a change in binding to be useful, the affinity of the active site for G414 must not also weaken by an order of magnitude between the first and second steps of splicing. This seems likely, because there are interactions in the second step of splicing not present in the first that are expected to enhance the affinity for G414 (23). Furthermore, if the energetic contribution for coupling is intrinsic to the reactive phosphate, then the affinity for G414, with the 3′-splice site-reactive phosphate linked to it, may not be decreased in the presence of a cleaved 5′ exon.

We thank Rob Kuchta and Doug Turner for helpful comments on the manuscript. This work was supported in part by National Institutes of Health Grant GM28039 to T.R.C. and a grant from the Lucille P. Markey Charitable Trust to D.H., who is a Lucille P. Markey Scholar in Biomedical Science. T.R.C. is an investigator of The Howard Hughes Medical Institute and an American Cancer Society Professor.

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