Contributions of 2′-Hydroxyl Groups of the RNA Substrate to Binding and Catalysis by the *Tetrahymena* Ribozyme. An Energetic Picture of an Active Site Composed of RNA†

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ABSTRACT: The ribozyme derived from the intervening sequence of *Tetrahymena thermophila* pre-rRNA catalyzes a site-specific endonuclease reaction with both RNA and DNA oligonucleotides: CCCUC-UAAAAA + G ⇌ CCCUCU + GAAAAA. However, the RNA substrate (rS) binds ~10^4-fold stronger than the DNA substrate (dS) and once bound reacts ~10^4-fold faster. Here we have investigated the role of individual 2′-hydroxyl groups by comparing the binding and reactivity of "chimeric" oligonucleotide substrates, in which the 2′-substituents of the individual sugar residues have been varied. Chimeric substrates containing a single ribonucleotide at positions −6 to +3 (numbered from the cleavage site) were cleaved faster than dS by factors of 3.5, 3.5, 2.3, 65, 18, 1700, 7.8, 1.7, and 1.4 [$(k_{cat}/K_m)^{chimeric\ S}/(k_{cat}/K_m)^{dS}$]. The sum of the energetic contributions from the individual 2′-hydroxyl groups of 13.3 kcal/mol accounts for the 12.2 kcal/mol greater stabilization for RNA than for DNA in binding and cleavage (i.e., overall transition-state stabilization). This observation and the significant energetic effects from single ribose substitutions at positions −3 to +1 strongly suggest that local interactions, rather than overall helical differences, largely account for the different binding and reactivity of the DNA and RNA substrates. Each 2′-hydroxyl group was evaluated for its effect on each of three reaction steps leading to the chemical transition state: two binding steps (duplex formation and docking into tertiary interactions) and the chemical cleavage step. The 2′-hydroxyl groups at positions −3 and −2 stabilize docking, and this stabilization is maintained in the chemical step. This "uniform binding" indicates that these interactions contribute to catalysis by positioning the oligonucleotide substrate for reaction. The 2′-hydroxyl at position +1 has a small effect on the binding step and an additional small but significant effect on the chemical step. Thus, the ribozyme, like protein enzymes, can take advantage of interactions away from the site of chemistry to provide stabilization specifically in the transition state. The 2′-hydroxyl at position −1 exerts its large effect nearly exclusively on the chemical step [Herschlag, D., Eckstein, F., & Cech, T. R. (1993) *Biochemistry* (following paper in this issue)]. The energetic effects of other modifications of the 2′-substituents provide a crude picture of the active site. The 2′-OCH_3 substituent at position −3 inhibits the reaction ~10-fold relative to 2′-H, suggesting that an unfavorable interaction cannot be avoided by an isoenergetic structural rearrangement. Furthermore, this binding pocket of the ribozyme has a high degree of specificity: 2′-F, -NH_2, and -NH_3^+ are also ineffective substitutes for the 2′-OH moiety at position −3, even though these substituents lack the steric bulk of the *O*-methyl group. These effects suggest that this binding site composed of RNA has some rigidity and can discriminate between substrates at the level of single functional groups.

The RNA enzyme or "ribozyme" derived from the self-splicing intron of *Tetrahymena thermophila* pre-rRNA catalyzes a site-specific endonuclease reaction with both RNA and DNA oligonucleotides (eq 1; Zaug et al., 1986, 1988; Herschlag & Cech, 1990c; Robertson & Joyce, 1990).

$$\overset{-5\ -3\ -1\ +2}{\text{CCCUCUAAAAA}} + \text{G} \rightleftharpoons \text{CCCUCU} + \text{GAAAAA} \quad (1)$$
$$\text{(S)} \qquad\qquad \text{(P)}$$

S has a sequence analogous to that adjoining the 5′ splice site of the pre-rRNA and is recognized by base pairing to the 5′ exon binding site of the ribozyme to form the P1 duplex (Figure 1). Even though the RNA and DNA oligonucleotides can form the same base pairs in the P1 duplex, the DNA substrate binds to the ribozyme ~10^4-fold weaker than the RNA substrate and once bound reacts ~10^4-fold slower (Herschlag & Cech, 1990a,c). This corresponds to a difference of 12.2 kcal/mol in total transition-state stabilization (10 mM $MgCl_2$, 50 °C). There are two limiting models for this difference: (1) localized interactions that involve one or more of the 2′-hydroxyl groups of the RNA substrate are missing with the DNA substrate; or (2) overall geometrical differences between the duplex containing the RNA oligonucleotide substrate and the 5′ exon binding site of the ribozyme (Figure 1) and that containing the DNA oligonucleotide allow the RNA substrate to fit into the active site better than the DNA substrate. The observations that RNA oligonucleotides bind ~10^4-fold stronger than predicted for simple base pairing interactions with the 5′ exon binding site and that DNA oligonucleotides bind about as strongly as predicted for simple duplexes

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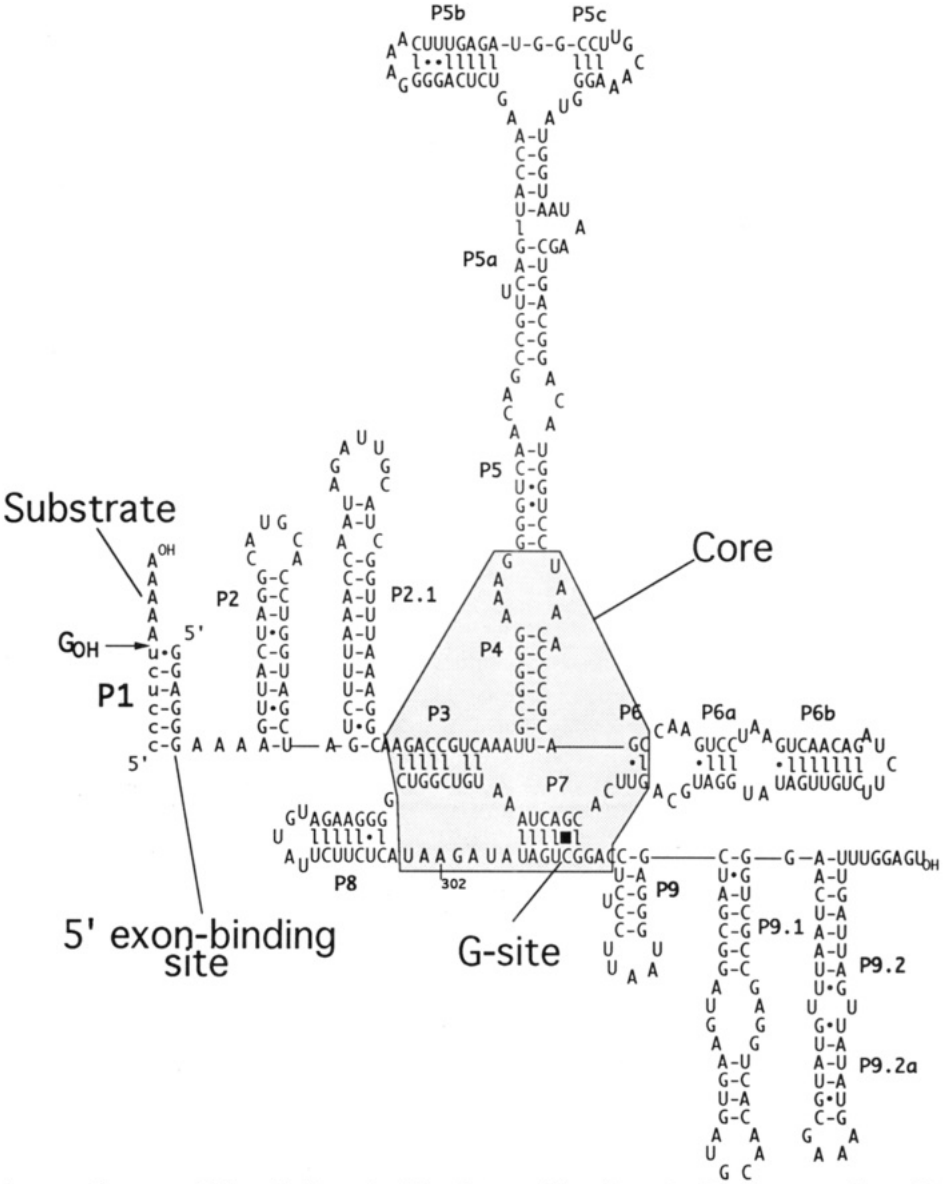


FIGURE 1: The *Tetrahymena* ribozyme with bound oligonucleotide substrate. The oligonucleotide (lower-case letters) base pairs with the 5′ exon binding site of the L-21 *Sca*I ribozyme to form duplex P1. Evidence for this base pairing arises from phylogenetic comparisons, mutational studies, and binding studies with oligonucleotide variants (Cech & Bass, 1986; Cech, 1990). "Core" is the catalytic core of the ribozyme, as defined by sequence conservation (Michel & Westhof, 1990), mutational sensitivity (Couture et al., 1990), and inaccessibility to solvent-based cleavage agents (Latham & Cech, 1989); it includes the G-site that binds the guanosine nucleophile (Michel et al., 1989). Residue A302 in J8/7, the joining region between helices P7 and P8, has been implicated in a tertiary interaction with the 2′-hydroxyl of U(−3) of the oligonucleotide substrate (numbering from eq 1; Pyle et al., 1992).

(Sugimoto et al., 1989; Herschlag & Cech, 1990b,c; Pyle et al., 1990) led us to favor the first model involving specific interactions with one or more 2′-hydroxyls. Here we test these models. The results strongly favor local effects of the 2′-hydroxyl groups, though it is not possible in all cases to differentiate between direct and indirect effects.

The near independence of the energetic effects has allowed separation of the effects from each 2′-hydroxyl group into contributions in individual steps. The results strongly suggest that the tertiary binding interactions are maintained in the transition state for cleavage. These energetic effects on binding are also similar to those for the oligonucleotide product (P, eq 1), reported by Pyle and Cech (1991) and by Bevilacqua and Turner (1991). Thus, the tertiary interactions position both the oligonucleotide substrate and product for reaction. Finally, comparison of the effects of a series of 2′-substituents [2′-H (deoxyribo), -OH (ribo), -OCH_3, -F, and -amino] at some positions within the oligonucleotide has led to a crude energetic picture of the active site, showing that a binding site composed of RNA can provide significant discrimination at the level of an individual functional group. In the following paper these substituents have been employed at position −1 to obtain a view of transition-state interactions at the site of bond cleavage (Herschlag et al., 1993).

MATERIALS AND METHODS

Materials. Ribozyme was prepared by *in vitro* transcription and purified as described previously (Zaug et al., 1988). Commercial ribonucleotide phosphoramidites were from ABN (Biogenics); 2′-*O*-methyl phosphoramidites were from Chem-Genes (Needham, MA). 2′-Fluoro-2′-deoxyuridine and its phosphoramidite were synthesized as described for 2′-fluoro-2′-deoxythymidine (Williams et al., 1991). 2′-Amino-2′-deoxyuridine was prepared as the trifluoroacetyl derivative as described (Imazawa & Eckstein, 1979); its phosphoramidite was prepared as described by Williams et al. (1991). Other

materials were as described previously (Herschlag, 1992).

Oligonucleotide Synthesis. All oligonucleotides were made by solid-phase synthesis using an Applied Biosystems 380B DNA synthesizer. Coupling times for all residues other than deoxyribonucleosides were increased from the standard 1 min for DNA synthesis to 10 min. All other parameters were unchanged. Oligonucleotides containing ribose residues were deprotected in ethanol/30% NH_4OH (1:3) overnight at 55 °C and dried in vacuo three times; other oligonucleotides were deprotected in 30% NH_4OH. Oligonucleotides not containing ribose residues were dissolved in 10 mM Tris/1 mM EDTA, pH 7.5, and 5′-end-labeled directly. For oligonucleotides containing ribose residues, the 2′-silyl protecting group was removed by treatment with 1.0 M tetrabutylammonium fluoride in tetrahydrofuran overnight at room temperature. An aliquot from this solution was then diluted ~100-fold into aqueous solution, from ~2 mM oligonucleotide to a final concentration of ~10 μM in a kinase reaction mixture (see below).

This procedure requires no purification step until after 5′-end-labeling. The only purification step in the procedure is gel electrophoresis of the 5′-end-labeled oligonucleotide. This greatly facilitates processing large numbers of oligonucleotide samples and is possible because only a small amount of S is required for single-turnover kinetic experiments ([S] ≪ [E]). Reactions have typically been followed for multiple half-lives (see Kinetics below), so a minor fast-reacting contaminant would not have a significant effect on the observed rate constants. An important control was demonstration that the concentration of S does not affect the observed rate constant for its first-order disappearance, indicating that there are no contaminants of S that are affecting the reaction.

5′-End-Labeling and Purification of Oligonucleotides. Oligonucleotides were 5′-end-labeled with approximately equimolar [γ-^{32}P]ATP using T4 polynucleotide kinase, as described previously (Zaug et al., 1988), and purified from unlabeled material and oligonucleotide side products of the synthesis by electrophoresis on a nondenaturing 24% polyacrylamide gel. The band corresponding to the full-length, deprotected oligonucleotide (see below) was excised from the gel, and the oligonucleotide was eluted overnight at 4 °C into 10 mM Tris/1 mM EDTA, pH 7.5. Oligonucleotides were used directly without further purification, and the concentration of labeled oligonucleotide was estimated from its specific activity. Control experiments showed that oligonucleotides purified by this procedure gave the same rate constants as oligonucleotides that were ethanol precipitated subsequent to elution. In addition, the concentration of labeled oligonucleotide used in single-turnover experiments did not affect the observed rate, provided that the condition [S] ≪ [E] was maintained.

The oligonucleotide of interest was preliminarily identified by its migration relative to 5′-end-labeled standards of the all deoxyribo- and/or all ribo-oligonucleotide (synthesized by automated synthesis and by *in vitro* T7 transcription, respectively, and sequenced previously). Typically, there were additional minor bands both with slower mobility (presumably non-deprotected material) and with faster mobility (presumably truncated and breakdown products) than the candidate band. The candidate band for each oligonucleotide was characterized for the number of nucleotides by partial digestion with P1 nuclease and for the positions of non-ribose residues by resistance to alkaline degradation. In all cases the candidate band had the correct length and the correct position of non-ribose residues. In addition, 5′-end-labeled oligonucleotides with single ribose residues gave a single band of the expected migration following digestion with RNase T2.

Alkaline Hydrolysis. It is worth noting that a modification of the standard alkaline hydrolysis method [which entails boiling RNA samples in carbonate buffer (Donis-Keller et al., 1977)] was employed. The oligonucleotides were simply treated with 0.2 N NaOH at 37 °C for 30 min and reactions quenched by addition of ~1 equiv of $TrisH^+$. Thus, reactions can be carried out in Eppendorf tubes rather than sealed capillary tubes, as is required for the standard protocol. For the oligonucleotides that were investigated, the ladders were at least as uniform as typically observed for the standard method. (For considerably longer RNA molecules, less NaOH, lower temperature, and/or shorter times would be required to avoid overdigestion.)

Kinetics. All reactions were single turnover, with ribozyme (E) in excess of 5′-labeled oligonucleotide (S),[1] and were carried out at 50 °C in 10 mM $MgCl_2$ and 50 mM sodium MES, pH 7.0 [determined at 25 °C; pH 6.7 at 50 °C, calculated from Good et al. (1966)], or 50 mM sodium MES, pH 5.2 [determined at 25 °C; pH 4.9 at 50 °C, calculated from Good et al. (1966)], as described previously (Herschlag & Cech, 1990a) and in the tables. Briefly, reactions were initiated by addition of 5′-end-labeled S after a 15-min preincubation of E, $MgCl_2$ buffer, and G at 50 °C. Typically, 6 or 7 aliquots of 1–2 μL were removed from 20-μL reaction mixtures at specified times and quenched with ~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. P and S were separated by electrophoresis on 20% polyacrylamide/7M urea gels, and their ratio at each time point was quantitated with use of an Ambis radioanalytic scanner or Molecular Dynamics Phosphorimager. Reactions were followed for ~$3t_{1/2}$ or, for the slower reactions, for 210 min so that rate constants were determined by the first-order disappearance of S or by the initial rate of formation of P. End points of ~95% were used to account for ~5% unreactive starting material. For the slower reactions, there was no evidence of curvature from inactivation of E over the 210 min time course.

Description of Kinetic Constants. $(k_{cat}/K_m)^S$ represents the observed second-order rate constant for the reaction: E·G + S → products, and $(k_{cat}/K_m)^G$ represents the second-order rate constant for the reaction: E·S + G → products. "$(k_{cat}/K_m)^S$ conditions" were established by showing that the conversion of labeled S to P was first order, that the reaction was first order in E (i.e., the observed rate constant for reaction of S increased linearly with [E]), and that this observed rate

[1] The following abbreviations are used to specify the oligonucleotides used herein: S, CCCUCUAAAAA, meant generically to represent this sequence with the identity of the sugar residues not specified; P, CCCUCU. To specify the identity of the sugar residues, the following nomenclature is employed: rS and dS refer to all ribose and all deoxyribose oligonucleotides, respectively. Chimeric oligonucleotides are named in accord with the residues that are different from the ribose "background" of rS or the deoxyribose "background" of dS. For example, –3r,dS signifies a ribose residue at position –3 (eq 1) in the deoxyribose background, i.e., with deoxyribose residues at all other positions: dCdCdCrUdCdUdAdAdAdAdA; likewise, –3d,rS signifies a deoxyribose residue at position –3 in the ribose background: rCrCrCdUrCrUrArArArArA. Other sugar derivatives are signified as follows: m, 2′-methoxy-2′-deoxyribose; F, 2′-fluoro-2′-deoxyribose; N, 2′-amino-2′-deoxyribose. The following abbreviations are also used: E, ribozyme; G, guanosine; P1, the duplex between S (or P) and the 5′ exon binding site of the ribozyme (Figure 1); Tris, tris(hydroxymethyl)aminomethane; MES, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid.

Table I: Comparison of the Reactions of Ribo- and Deoxyribo-pCCCUCUAAAAA (rS and dS)[a]

substrate	k_{on}^S (M^{-1} min^{-1})	k_{off}^S (min^{-1})	K_d^S (nM)	k_c (min^{-1})	k_c/K_d^S (M^{-1} min^{-1})
rS	1.5×10^8 [b]	0.35[c]	2[d]	~200[e]	$\sim 1.0 \times 10^{11}$
dS[f]			30 000	0.018	600

[a] 50 °C, 50 mM sodium MES, pH 7, and 10 mM $MgCl_2$ (see Materials and Methods). [b] For rS, k_{on}^S, the rate constant for binding of S to the ribozyme, has been shown to equal $(k_{cat}/K_m)^S$ (Herschlag & Cech, 1990a). $(k_{cat}/K_m)^S$ was obtained with 5–10 nM ribozyme, ~1 nM 5′-end-labeled S, and 2 mM G. This value is the same, within ~40%, as that previously reported for the oligonucleotide substrate containing two 5′G residues from experiments with a different ribozyme preparation (Herschlag & Cech, 1990a). [c] The value of k_{off}^S, the rate constant for dissociation of S from the ribozyme, was obtained from pulse–chase experiments with 0–2 μM G as described in Materials and Methods. [d] $K_d^S = [E][S]/[E{\cdot}S] = k_{off}^S/k_{on}^S$. [e] Estimated from $(k_{cat}/K_m)^G = 1 \times 10^6$ M^{-1} min^{-1} for reaction of E·S* + G → P* (Table V) and the value of $K_d^G = 200$ μM (McConnell et al., in press), such that $k_c = (k_{cat}/K_m)^G \times K_d^G$, as described previously (Herschlag & Cech, 1990a; Herschlag et al., 1991). It should be noted that the value of K_d^G used in this estimate is 5-fold lower than K_d^G = 1 mM used in the small extrapolation to saturating G in the reaction of the DNA substrate (Herschlag & Cech, 1990c). This difference arises from a 5-fold coupling between binding of RNA substrates and G, but not DNA substrates and G (McConnell et al., in press). Modest errors from the uncertainties in the calculated value of k_c for the RNA substrate would not affect the conclusions herein. [f] From Herschlag and Cech (1990c), except that the $(k_{cat}/K_m)^S$ measurement was repeated with the ribozyme and buffer preparations used in these experiments; a value in reasonable agreement with the previous value was obtained (~50% larger). The dissociation constant for DNA oligonucleotides, obtained from inhibition, was previously shown to be the same with several different ribozyme preparations (Young et al., 1991; D.H. and B. Young, unpublished results). Because the reaction is slow, it is assumed that the chemical step is rate-limiting for $(k_{cat}/K_m)^S$ so that $(k_{cat}/K_m)^S = k_c/K_d^S$ and $K_i^S = K_d^S$ (Herschlag & Cech, 1990c). Further data supporting this assumption are described in the next paper (Herschlag et al., 1993).

constant was independent of [G] (i.e., that G was saturating with respect to E). "$(k_{cat}/K_m)^G$ conditions" were established by showing that the conversion of labeled S to P was first order, that the reaction was zero order in E (i.e., that the observed rate constant was unaffected by changes in [E]), and that the reaction was first order in G (i.e., the observed rate constant increased linearly with [G]).

The rate constant k_c represents the first-order rate constant for reaction of the ternary complex: E·S·G → products. This rate constant has been calculated as described in Table I.

Values of k_{off}^S were determined in pulse–chase experiments (Rose et al., 1974), as described previously (Herschlag & Cech, 1990a), except that 2-fold greater volumes were used to facilitate manipulations. Briefly, the rationale of this experiment is to generate E·S* (where S* represents labeled S) and then challenge this complex with G and a large excess of unlabeled oligonucleotide. Thus, there is a competition between reaction of E·S* (k_c^{app}) and dissociation of S* from E·S* (k_{off}^S) such that the value of k_{off}^S can be determined both from (1) the fraction of S* converted to product $[=k_c^{app}/(k_c^{app} + k_{off}^S)]$, with k_c^{app} determined in independent experiments with saturating E and the appropriate concentration of G, and (2) the observed rate constant for this conversion $[=k_c^{app} + k_{off}^S$; see Herschlag & Cech (1990a) for a more detailed description]. The concentration of G was varied depending on the oligonucleotide substrate so that k_c^{app} and k_{off}^S were similar, thereby giving the greatest precision. Values of k_{off}^S were obtained from independent experiments with varying concentrations of G. It should be noted that there is cleavage of S in the absence of G so that some of the determinations of k_{off}^S for the fast-reacting and slow-dissociating substrates were obtained in the absence of added G (Herschlag & Cech, 1990a). Control experiments in which both the labeled and unlabeled oligonucleotide were added with G gave no significant cleavage of S*, demonstrating that the "chase" was effective.

The rate constant for S binding to the ribozyme, k_{on}^S, is equal to $(k_{cat}/K_m)^S$ when all S bound to E is cleaved rather than dissociates in a pulse–chase experiment (Herschlag & Cech, 1990a). In the current study, k_{on}^S was shown to be equal to $(k_{cat}/K_m)^S$ for several oligonucleotides in the ribose background (data not shown; see Table V below).

Estimation of Error Limits. As observed previously, there were variations in rate constants of up to ~2-fold from experiment to experiment (Herschlag & Cech, 1990a). However, the ratios of rate constants within an experiment, which are used in the analyses herein, varied less. Error estimates are based on the range of the ratios observed in independent experiments.

RESULTS

Energetic Differences in the Reactions of RNA and DNA Substrates. We previously compared the reactions of deoxy-CCCUCUAAAAA and ribo-GGCCCUCUAAAAA, where the 5′-terminal G residues were present to allow synthesis by transcription using T7 RNA polymerase (Herschlag & Cech, 1990c). Previous binding studies with RNA oligonucleotide products (e.g., P in eq 1) revealed only a small decrease in binding affinity of ~3–5-fold upon removal of the 5′-GG (Herschlag & Cech, 1990b), and the addition of deoxy-GG to a DNA oligonucleotide product (i.e., deoxy-CCCUCU) had a similar small effect (D.H., unpublished results). However, to ensure that all differences observed in the present study could be ascribed to the 2′ substituents and not the 5′-terminal G residues, rate and equilibrium constants for reaction of ribo-CCCUCUAAAAA (rS)[1] were obtained to allow direct comparison with dS (Table I). The kinetic and thermodynamic parameters for rS are the same as those for the ribo substrate containing 5′-GG, except that k_{off}^S is ~2-fold faster with the G residues removed. The transition state for the chemical step of the reaction: E·G + S → [E·G·S]* is 12.2 kcal/mol more stable for rS than for dS, relative to the free ribozyme and substrates.[2]

Transition-State Effects from Single Ribose Substitutions. The reactions of chimeric ribo/deoxyribooligonucleotides, each containing a single ribonucleotide at positions −6 to +3, are compared to the reaction of dS[1] in Figure 2. These and other data were used to obtain the $(k_{cat}/K_m)^S$ values in Table II; $(k_{cat}/K_m)^S$ corresponds to the observed second-order reaction of E·G and S (see Description of Kinetic Constants in Materials and Methods). The values of $\Delta\Delta G^*$ obtained from the ratio of $(k_{cat}/K_m)^S$ values for the chimeric substrates relative to that for dS are plotted in Figure 3.

The following suggest that the chemical step is rate-limiting for $(k_{cat}/K_m)^S$ for all of these substrates so that the free energy difference monitored is that for the reaction: E·G + S → [E·G·S]*, where [E·G·S]* is the transition state for the actual chemical cleavage. (1) All of the $(k_{cat}/K_m)^S$ values for the chimeric substrates in Table II are well below 10^8 M^{-1} min^{-1},

[2] Obtained from the k_c/K_d^S values in Table I with use of the equation: $\Delta\Delta G^* = -RT \ln (k_{rS}/k_{dS})$. Throughout, the sign convention in the free energy comparisons uses dS as the standard so that reactions faster than that of dS are represented by positive values of $-\Delta\Delta G^*$. This relationship is obtained from the equation: $\Delta G^* = -RT \ln (kh/k_BT)$, with k in units of s^{-1} (or s^{-1} M^{-1} for second-order reactions with a standard state of 1 M reactants), where R = 1.987 cal/(mol·K), T = 323 K (50 °C), h = 1.58×10^{-34} cal s, and $k_B = 3.30 \times 10^{-24}$ cal K^{-1}.

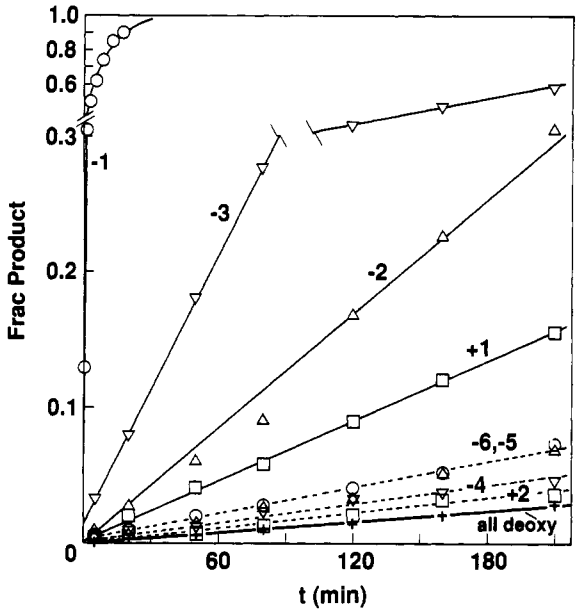


FIGURE 2: Reactions of a series of chimeric ribo/deoxyribo-oligonucleotide substrates (pCCCUCUAAAAA) each containing a single ribose residue at positon –6 (- -O- -), –5 (- -Δ- -), –4 (- -▽- -), –3 (▽), –2 (Δ), –1 (O), or +2 (- -□- -), compared to the reaction of dS (+; thick line). Reaction of the substrate with ribose at position +3 (+3r,dS) is omitted for clarity; data points fell between those for dS and +2r,dS. The fraction of P (pCCCUCU) formed as a function of time is plotted; no other products were observed. Reactions were single turnover with 300 nM ribozyme, ~5 nM 5′-end-labeled S, and 2 mM G in the presence of 50 mM sodium MES, pH 7, and 10 mM $MgCl_2$ at 50 °C.

Table II: Effect of Single Ribose Substitutions on Total Transition-State Stabilization[a]

substrate[b]	k_{rel}[c]	$-\Delta\Delta G^{\ddagger}$[d] (kcal/mol)	substrate[b]	k_{rel}[c]	$-\Delta\Delta G^{\ddagger}$[d] (kcal/mol)
dS	(1)	(0)	+1r,dS	7.8	1.3
–6r,dS	3.5	0.8	+2r,dS	1.7	0.3
–5r,dS	3.5	0.8	+3r,dS	1.4	0.2
–4r,dS	2.3	0.5	total		13.3 (±1.5)[e]
–3r,dS	65	2.7	rS		12.2[f]
–2r,dS	18	1.9			
–1r,dS	1700	4.8			

[a] From single-turnover reactions at 50 °C, 50 mM sodium MES, pH 7, with 2 mM G, 300–1000 nM ribozyme, and ~5 nM 5′-end-labeled oligonucleotide substrate. [b] Substrate abbreviations as defined in footnote 1 in the text. [c] $k_{rel} = (k_{cat}/K_m)^{chimeric\ S}/(k_{cat}/K_m)^{dS}$. All values of k_{rel} were obtained in side-by-side comparisons with dS. The rate for each reaction was determined to be linear with respect to ribozyme concentration, confirming that $(k_{cat}/K_m)^S$, the second-order rate constant for the reaction E·G + S* → products, was being followed (see Description of Kinetic Constants in Materials and Methods). Error estimates for these data are shown in the plot of $\Delta\Delta G^{\ddagger}$ in Figure 3. Each value was determined in at least two independent experiments, each with reactions at more than one ribozyme concentration. Typical data are shown in Figure 2. [d] Determined at 50 °C from the equation $-\Delta\Delta G^{\ddagger} = RT \ln k_{rel}$ as described in footnote 2 in the text. Positive values of $-\Delta\Delta G^{\ddagger}$ represent faster reactions of the chimeric substrate relative to dS. [e] The error estimate represents a cumulative value from an uncertainty of ~40% in each value of k_{rel} and does not assume that uncertainty is random. The given error estimate is therefore larger than the estimate that would be obtained by assuming random uncertainty. [f] From Table I, as described in the text.

the rate constant determined for binding of several different oligonucleotide substrates to the ribozyme [Herschlag & Cech (1990a) and Table V below; the largest value of $(k_{cat}/K_m)^S$ in this series of chimeric substrates is $\sim 10^6$ M^{-1} min^{-1} for –1r,dS (from Figure 2 and related data)]. This makes it unlikely that binding of the oligonucleotide could be rate-limiting. (2) $(k_{cat}/K_m)^S$ for the fastest chimeric substrate,

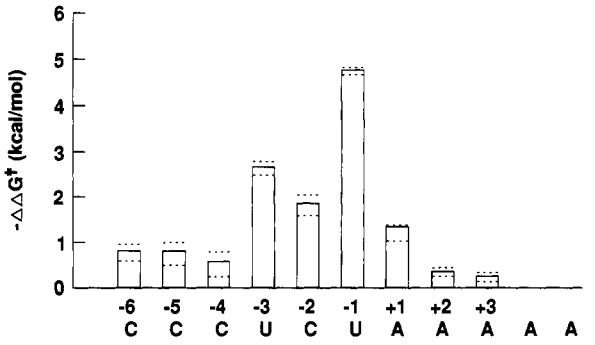


FIGURE 3: Transition-state stabilization by individual ribose residues. Deoxyribose residues of dS were individually replaced with ribose residues (i.e., substrates from Figure 2), and the effect was determined for the reaction: E·G + S → [E·G·S]* → products. The values of $-\Delta\Delta G^{\ddagger}$ represent the extent of transition-state *stabilization* from a single ribose substitution at the specified position relative to the reaction of dS. Values are from Table II, and errors (dashed lines) are estimated from the range of observed values.

–1r,dS, follows the same pH dependence as does $(k_{cat}/K_m)^S$ for dS (Herschlag et al., 1993), suggesting that the same step is rate-limiting. The substrate –3r,dS also follows the same pH dependence (Figure 5 below). (3) The thio effect on $(k_{cat}/K_m)^S$ on cleavage of the (R_P)-phosphorothioate of dS is the same, within error, as that for the chemical step for reaction of rS and is of the magnitude expected for rate-limiting chemical cleavage (Herschlag et al., 1991; J. A. Piccirilli and T.R.C., unpublished results). (4) The total transition-state free energy (binding and chemical step) gained from addition of individual 2′-hydroxyl groups to dS is similar to the free energy lost from removal of the corresponding 2′-hydroxyl groups from rS (Figure 6 below). (5) Further evidence that the actual chemical cleavage is indeed rate-limiting is presented in the following paper [Herschlag et al., 1993; see also Herschlag et al. (1991)].

The stabilization of 1.9–4.8 kcal/mol from the 2′-hydroxyl groups at positions –3, –2, and –1 is considerably greater than the modest stabilization of 0.5–0.8 kcal/mol from addition of 2′-hydroxyl groups at positions –6, –5, and –4, the other base-paired positions. This suggests that the effects at –3 to –1 are unlikely simply to reflect duplex stability. In addition, the limited data on DNA/RNA hybrid duplexes suggest that there can be modest destabilization relative to RNA/RNA duplexes (Martin & Tinoco, 1980; Nelson & Tinoco, 1982; Walker, 1988; Bevilacqua & Turner, 1991; Hall & McLaughlin, 1991), but extensive destabilization has only been observed with deoxyA/riboU pairs (Martin & Tinoco, 1980), which are not present in the P1 duplex (Figure 1).

In the non-base-paired region, the 2′-hydroxyl at position +1 gives significant transition-state stabilization, whereas 2′-hydroxyls at positions +2 and +3, more remote from the cleavage site, provide negligible contributions.

2′-O-Methyl Substitutions Help Distinguish Effects at the Individual Positions. The 2′-methoxy substituent favors the same sugar conformation as the 2′-hydroxyl of ribose (Uesugi et al., 1979; Guschlbauer & Jankowski, 1980), but is larger and lacks the ability to donate a hydrogen bond. A series of chimeric oligonucleotides with single 2′-*O*-methyl residues at each of the positions –6 to +3 and all of the remaining residues deoxyribose were tested as substrates (Table III). The effects of the single 2′-*O*-methyl substitutions are compared to those from the corresponding 2′-hydroxyl substitutions in Figure 4. At positions –6, –5, and –4 there are similar small effects from the 2′-OCH_3 and -OH substituents. In contrast, the opposing effects from hydroxyl and *O*-methyl substitution at

Table III: Effect of Single 2′-*O*-Methyl-2′-deoxyribose Substitutions on Total Transition-State Stabilization[a]

substrate[b]	k_{rel}[c]	$-\Delta\Delta G^{\ddagger}$[d] (kcal/mol)	substrate[b]	k_{rel}[c]	$-\Delta\Delta G^{\ddagger}$[d] (kcal/mol)
dS	(1)	(0)	−2m,dS	2.2	0.5
−6m,dS	4.3	0.9	−1m,dS	<0.06[e]	<(−1.8)
−5m,dS	3.0	0.7	+1m,dS	4.0	0.9
−4m,dS	2.7	0.6	+2m,dS	1.8	0.4
−3m,dS	<0.1[e]	<(−1.5)	+3m,dS	1.2	0.1

[a] From single-turnover reactions at 50 °C, 50 mM sodium MES, pH 7, with 2 mM G, 300–1000 nM ribozyme, and ~5 nM 5′-end-labeled S. [b] Substrates as defined in footnote 1 in the text. [c] $k_{rel} = (k_{cat}/K_m)^{chimeric\ S}/(k_{cat}/K_m)^{dS}$. All values of k_{rel} were obtained in side-by-side comparisons with dS. The rate for each reaction was determined to be linear in ribozyme concentration, confirming that $(k_{cat}/K_m)^S$, the second-order rate constant for the reaction E·G + S → products, was being followed (see Description of Kinetic Constants in Materials and Methods). [d] Determined at 50 °C from the equation $-\Delta\Delta G^{\ddagger} = RT \ln k_{rel}$ as described in footnote 2 in the text. Positive values of $-\Delta\Delta G^{\ddagger}$ represent faster reactions of the chimeric substrate relative to dS. [e] The symbol "<" represents a limit of detection; no reaction was observed for these substrates.

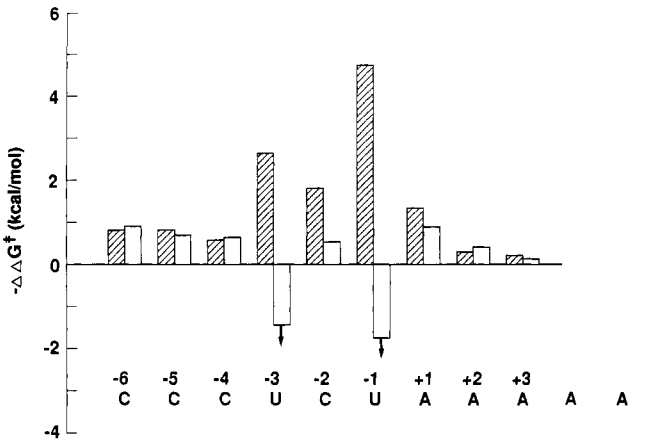


FIGURE 4: Comparison of transition-state stabilization from individual 2′-OH (shaded) and 2′-OCH_3 (open) substitutions. Deoxyribose residues of dS were individually replaced with ribose or 2′-*O*-methyl-2′-deoxyribose residues, and the effect was determined for the reaction: E·G + S → [E·G·S]‡ → products. $-\Delta\Delta G^{\ddagger}$ as defined in the legend to Figure 3; values for the single ribose substitutions are from Table II, and those for the single 2′-*O*-methyl-2′-deoxyribose substitutions are from Table III.

positions −3 and −1 suggest specific interactions with the hydroxyl groups at these positions. The effect at position −3 is pursued further below, and that at −1 is the subject of the following paper (Herschlag et al., 1993). Position −2 gives an intermediate result: 2′-OCH_3 and -OH both enhance transition-state stabilization, but the hydroxyl substituent has a larger effect.

Other 2′-Substituents at U(−3). To learn more about the energetic effect at position −3, additional 2′-substituents were investigated (Table IV). In order to interpret the results with −3N,dS, it was necessary to know the protonation state of the 2′-amino group. The pK_a of the 2′-amino group of the dinucleotide, 5′-*O*-thymidinyl-3′-*O*-(2′-aminouridyl) phosphate, was determined to be 6.2 (±0.2) by ^{13}C NMR spectroscopy (F.E., unpublished results). This pK_a value strongly suggests that the amino group of −3N,dS in solution is deprotonated (i.e., $-NH_2$) under the standard reaction conditions of pH 7.

However, the protonation of the amino group could be different at the ribozyme active site in the transition state for the reaction. The pH dependence for cleavage of −3N,dS was therefore compared to that for cleavage of −3r,dS (Figure 5; for the reaction: E·G + S (+H^+) → [E·G·S(H^+)]‡, where the parentheses around the proton indicate that it may or may not participate in the reaction). If the amino group were protonated in the transition state, then the rate constant for reaction of −3N,dS would be expected to decrease with increasing pH *relative to* oligonucleotides lacking the amino group. This would give a slope of −1 in Figure 5B (dashed line). The observed slope of zero in Figure 5B therefore indicates that the reaction of −3N,dS proceeds without gain of a proton over the pH range of ~6–9, so the reactive species at pH 7 is the -NH_2 form.[3]

Nevertheless, −3N,dS with a protonated amino group could be the reactive species at low pH. The pK_a of ~6 of this amino group and the absence of positive deviation at low pH in Figure 5B can be used to obtain an approximate upper limit for the rate of reaction of the -NH_3^+ species. If the -NH_3^+ form of −3N,dS reacted 10-fold faster than the -NH_2 form, a positive deviation of 4-fold would be expected at pH ~6 (since the amino group would be half-protonated: $k_{rel}^{predicted} = 10 \times 0.5 + 1 \times 0.5 = 6$, where 10 and 1 represent the assumed relative rate constants for -NH_3^+ and -NH_2, respectively, and 0.5 represents the fraction of each species present at pH ~6). The absence of such a positive deviation provides an *upper limit* for the reactivity of −3N,dS(-NH_3^+) of ~10-fold greater than that of −3N,dS(-NH_2) (Table IV).

Table IV: Effect of 2′-Substituents at U(−3) on Total Transition-State Stabilization[a]

2′-substituent[b]	k_{rel}[c]	$-\Delta\Delta G^{\ddagger}$[d] (kcal/mol)	2′-substituent[b]	k_{rel}[c]	$-\Delta\Delta G^{\ddagger}$mo[d] (kcal/mol)
H	(1)	(0)	F	0.5	−0.4
OH	65	2.7	NH_2[f]	0.4	−0.6
OCH_3[e]	<0.1	<(−1.5)	NH_3^+[f]	<4	<0.9
	[0.08]	[−1.6]			

[a] From single-turnover reactions at 50 °C, 50 mM sodium MES, pH 7, with 2 mM G, 300–1000 nM ribozyme, and ~5 nM 5′-end-labeled S. [b] Substituents are at the 2′ position of U(−3) of S (eq 1). All other positions had deoxyribose residues, so that the oligonucleotide referred to as "H" is dS, as "OH" is −3r,dS, as "OCH_3" is −3m,dS, and as "F" is −3F,dS. The value for "NH_2" and the limit for "NH_3^+" are from the pH dependence for reaction of −3N,dS (Figure 5 and Results). [c] $k_{rel} = (k_{cat}/K_m)^{chimeric\ S}/(k_{cat}/K_m)^{dS}$. All values of k_{rel} were obtained in side-by-side comparisons with dS, except for the value in square brackets (see footnote *e*). The rate for each reaction was determined to be linear in ribozyme concentration, confirming that $(k_{cat}/K_m)^S$, the second-order rate constant for the reaction E·G + S → products, was being followed (see Description of Kinetic Constants in Materials and Methods). Each value was determined in at least two independent experiments. [d] Determined at 50 °C from the equation $-\Delta\Delta G^{\ddagger} = RT \ln k_{rel}$ as described in footnote 2 in the text. Positive values of $-\Delta\Delta G^{\ddagger}$ represent faster reactions of the chimeric substrate relative to dS. [e] The symbol "<" here represents a limit of detection; no reaction was observed for this substrate (−3m,dS). Thus, the substrates −3m,−1r,dS and −1r,dS were compared to give the values in square brackets; the presence of the ribose at position −1 speeds the rate of cleavage. It is expected that the substituent at position −1 will not greatly perturb the effect at position −3, because the interactions are nearly independent (see Figure 6 and Discussion). In addition, the effect of the 2′-fluoro substituent at position −3 is the same whether there is a 2′-hydrogen or fluorine at position −1 (i.e., k_{rel} is the same from comparison of −3F,dS and dS and from comparison of −3F,−1F,dS and −1F,dS; data not shown). [f] The protonation state of the 2′-amino group was assigned from the pK_a of ~6 for the 2′-amino group (F.E., unpublished results) and the pH dependence of Figure 6, as described in the Results. The limit for -NH_3^+ was *estimated* from this pK_a and the pH dependence of Figure 5, as described in the Results. Note that this is an upper limit and in no way implies that a substrate containing 2′-NH_3^+ is more reactive than those containing -H, -F, or -NH_2.

[3] The unlikely mechanism involving loss of the proton of the 2′-hydroxyl group at position −3 is ruled out by the observation that the pH dependence is the same for dS and −3r,dS (Herschlag et al., 1993).

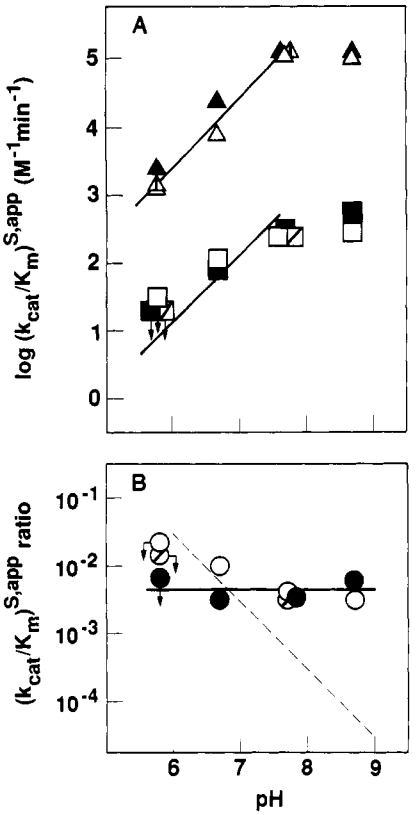


FIGURE 5: Effect of pH on the reactivity of oligonucleotide substrates with 2′-amino and 2′-hydroxyl substituents at U(–3). (A) Values of $(k_{cat}/K_m)^{S,app}$ for –3r,dS (triangles) and –3N,dS (squares). Different shading corresponds to independent experiments. (B) Ratio of these $(k_{cat}/K_m)^{S,app}$ values as a function of pH [k(–3N,dS)/k(–3r,dS)]. Each point represents data from a side-by-side comparison of the two substrates. The solid line in (A) has slope = 1; the solid line in (B) has slope = 0 and the dashed line slope = –1. Reactions were carried out with 2 mM G, 300–1000 nM ribozyme, and ~5 nM 5′-end-labeled substrate. The value of k_{obsd} was shown to be linear in ribozyme concentration at pH 6.7 for dS and –3r,dS and at pH 7.7 for –3N,dS so that $(k_{cat}/K_m)^S$ is being followed (see Materials and Methods). It is assumed that binding of S does not increase at lower pH values so that $(k_{cat}/K_m)^S$ conditions continue to hold; preliminary experiments with rS and –1d,rS support this assumption (D.H., unpublished results). No correction to fully saturating G was made so that the values of $(k_{cat}/K_m)^{S,app}$ are apparent. [The observed value of K_m^G is 1 mM for the reaction of dS with subsaturating concentrations of ribozyme; there is evidence that K_m^G is equivalent to the dissociation constant for the E·G complex (Herschlag & Cech, 1990c; Herschlag et al., 1991; McConnell et al., in press), so the same value of K_m^G is also expected to obtain for reactions of –3r,dS and –3N,dS with subsaturating ribozyme.] The following buffers were used: sodium MES, pH 5.8 and 6.7; sodium EPPS, pH 7.7 and 8.7. [The pH values were determined at 25 °C and have been corrected to 50 °C (Good et al., 1966).] It should be emphasized that these data are presented to compare the behavior of the substrates; no attempt was made to control for potential salt- and buffer-specific effects. A preliminary interpretation of related pH data is presented in the following paper (Herschlag et al., 1993).

Thus, the 2′-NH_3^+ at position –3 lowers the reactivity by at least 16-fold relative to the 2′-OH substituent (Table IV).

Separating Effects on Binding from Effects on the Chemical Step. Because of the weak binding of dS (K_d^{dS} = 30 μM; Herschlag & Cech, 1990c), separation of the effects of individual 2′-hydroxyl groups into effects on the binding step and on the chemical step was accomplished by studying chimeric substrates with single deoxyribose residues in an otherwise all ribo-oligonucleotide substrate (Table V).

Rate constants for binding and for dissociation of the chimeric oligonucleotide substrates –3d,rS, –2d,rS, –1d,rS, and +1d,rS were measured, and the dissociation constants were calculated ($K_d^S = k_{off}^S/k_{on}^S$; Table V). Removal of the 2′-hydroxyl group at each of the positions –3, –2, –1, and +1 had an effect on binding, with the largest effect coming from position –3, farthest from the reaction site. In contrast, the sites closest to the site of reaction had larger effects on the chemical step [$(k_{cat}/K_m)^G$ and k_c(–G)], with the hydroxyl at position –1 exhibiting by far the largest effect and that at position –3 having no measurable effect.[4]

Figure 6 compares the total transition state *stabilization* obtained from adding a 2′-hydroxyl group to dS (to give, for example, –3r,dS) with the total transition-state *destabilization* from removing a 2′-hydroxyl from rS. The free energy contributions from the individual 2′-hydroxyl groups are similar whether the surrounding residues are ribose or deoxyribose. However, there are significant quantitative differences at positions –3 and –2, with addition of a ribose in the deoxyribose background contributing more energy than is lost by taking away the analogous 2′-hydroxyl in the ribose background.

Two methodological differences between the experiments in the ribose and deoxyribose backgrounds should be noted, though neither is expected to significantly alter the comparison shown in Figure 6. (1) In the deoxyribose background (Table II), the free energy for the reaction E·G + S → [E·G·S]‡ was determined, whereas in the ribose background (Table V), that for the reaction E + G + S → [E·G·S]‡ was determined. This difference was allowed because at saturating G reactions in the ribose background are too fast to measure and at subsaturating G reactions in the deoxyribose background are very slow so that rate determinations are less accurate. Nevertheless, the same vaues of $-\Delta\Delta G^‡$ are expected for the reaction E + G + S for oligonucleotide substrates in the deoxyribose background as for the reaction E·G + S because there is evidence for an absence of coupling between binding of oligonucleotide substrates in the deoxyribose background and G to the ribozyme (experiments with dS and –1r,dS; McConnell et al., in press). (2) The data in the ribose background (Table V) were collected at pH 5.2, rather than pH 7 as for the data in the deoxyribose background (Table II). The lower pH was used because reactions in the ribose background appear to be log linear with pH at the lower pH values, but appear to deviate from linearity at higher pH values (D.H., unpublished results). Thus, the similarity of the pH dependence in the ribose background at low pH to the pH dependence in the deoxyribose background at low and neutral pH (Herschlag et al., 1993; D.H., unpublished results) suggests that this comparison is appropriate. Although the comparison in the deoxyribose background could have been performed at low pH, the slowness of these reactions would have limited the precision.

[4] $(k_{cat}/K_m)^G$, the second-order rate constant for the reaction E·S + G → products (see Materials and Methods), represents the binding of G as well as the cleavage step subsequent to G binding. Thus, the effects attributed to the chemical step could in principle arise from differential effects of the 2′-hydroxyls on the binding of G. (The reactions were carried out with subsaturating G to slow the reactions so that they could be monitored.) However, the following argue strongly that the effect is exerted on the chemical step: (1) $(k_{cat}/K_m)^G$, which includes the G binding step, and k_c(–G), which represents the hydrolysis reaction *in the absence of G*, are affected similarly by the removal of 2′-hydroxyl groups from rS (Table V); (2) the individual 2′-hydroxyl groups have similar effects in the deoxyribose background (Figure 6 below), even though the experiments in the deoxyribose background were performed with G nearly saturating; and (3) there is evidence that substitution of a 2′-H at position –1 of rS to give –1d,rS has no affect on the equilibrium binding constant for G (McConnell et al., in press).

Table V: Effect of Individual 2′-Hydroxyl Groups on the Binding Step and on the Chemical Step in the Ribose–Substrate Background[a]

substrate	k_{on}^{S} [b] (10^8 M^{-1} min^{-1})	k_{off}^{S} [c] (min^{-1})	K_d^S [d] (nM)	$(K_d^S)_{rel}$ [e]	$k_c(-G)$ [f,g] (min^{-1})	$(k_{cat}/K_m)^G$ [g,h] (M^{-1} min^{-1})	$1/(k_{cat}/K_m)^G{}_{rel}$ [e]	$1/k_{rel}$ [i]	$-\Delta\Delta G^{\ddagger}$ [j] (kcal/mol)
rS	1.5	0.35 ± 0.15	2.3	(1)	0.11	8800	(1)	(1)	(0)
-3d,rS	1.5	4.5 ± 1.5	30	13	0.10	9000	1	13	1.6
-2d,rS	1.4	1.5 ± 0.4	11	5	0.07	6200	1.4	6.7	1.2
-1d,rS[k]	~1.4	1.0 ± 0.3	7	3	~4×10^{-4}	15	590	1880	4.8
+1d,rS	1.3	0.7 ± 0.2	5	2	0.06	2700	3.3	7.6	1.3
+1m,rS	1.5	0.5 ± 0.2	3	1–2[l]	0.08	3800	2.3	3.2	0.7

[a] All determinations were made at 50 °C in 10 mM $MgCl_2$ and 50 mM sodium MES, at pH 5 or 7, as specified. All reactions were performed side-by-side with reactions of rS, so that the relative rate and equilibrium constants are more accurate than the absolute values (see Materials and Methods). [b] $(k_{cat}/K_m)^S$ was determined with a series of ribozyme concentrations of 2–10 nM and ~1 nM 5′-end labeled S and 2 mM G at pH 7. Pulse–chase experiments showed that binding rather than a subsequent step is rate-limiting for all of the substrates except for -1d,rS [see Herschlag et al. (1993)] so that $(k_{cat}/K_m)^S = k_{on}^S$, the rate constant for binding of S to the ribozyme (see Description of Kinetic Constants in Materials and Methods), as described previously for rS (Herschlag & Cech, 1990a). [c] Values of k_{off}^S, the rate constant for dissociation of S from the ribozyme, were obtained from pulse–chase experiments with 0–20 μM G (pH 7) (see Materials and Methods). [d] $K_d^S = [E][S]/[E{\cdot}S] = k_{off}^S/k_{on}^S$. k_{off}^S is for the reaction E·S → E + S, obtained from experiments in the absence of G and with G concentrations well below saturating, whereas k_{on}^S is for E·G + S → E·G·S, obtained with saturating G. However, previous experiments have shown that the presence of bound G does not affect the value of k_{on}^S for rS (Herschlag & Cech, 1990a; McConnell et al., in press), so that this measure of K_d^S is expected to hold for binding of S to the free ribozyme. [e] Value for rS relative to each chimeric S: $(K_d^S)_{rel} = K_d^{chimeric\ S}/K_d^{rS}$ and $(k_{cat}/K_m)^G{}_{rel} = \{(k_{cat}/K_m)^G\}^{chimeric\ S}/\{(k_{cat}/K_m)^G\}^{rS}$. Note that the values of $(K_d^S)_{rel}$ and $1/(k_{cat}/K_m)^G{}_{rel}$ larger than 1 signify *weaker* binding and *slower* reaction, respectively, of chimeric S compared with rS. [f] Rate constant for the site-specific hydrolysis of S in the absence of G (Herschlag & Cech, 1990a) in the single-turnover reaction E·S → products in sodium MES, pH 5, with saturating ribozyme (see footnote *g*). [g] For most experiments 200 nM ribozyme was used, which is saturating for all of the oligonucleotides (from K_d^S values above and data not shown) except -3d,rS, which is ~90% saturated. The values of $k_c(-G)$ and $(k_{cat}/K_m)^G$ for -3d,rS have been corrected by 10% to account for this. [h] Second-order rate constant for attack by G in the single-turnover reaction E·S + G → products in sodium MES, pH 5, with saturating ribozyme (see footnote *g*). [i] $k_{rel} = \{(k_{cat}/K_m)^G/K_d^S\}^{chimeric\ S}/\{(k_{cat}/K_m)^G/K_d^S\}^{rS} = \{(k_{cat}/K_m)^G{}_{rel}/(K_d^S)_{rel}\}$, representing the process E + G + S → [E·G·S]‡, where the transition state is that for the chemical step. Greater transition-state stabilization for rS than for the chimeric substrate (relative to the free reactants) gives values of $1/k_{rel} > 1$. The components of k_{rel} can be understood as follows: $(1/K_d^S)$ reflects the energy in going from E + S to E·S, and $(k_{cat}/K_m)^G$ represents the energy in going from E·S + G to the transition state for the chemical step ([E·G·S]‡). The rate advantage or disadvantage k_{rel} is hypothetical, because in the actual reaction binding of the oligonucleotide becomes rate-limiting, obscuring the chemical transition state. [j] $-\Delta\Delta G^{\ddagger} = RT\ln(1/k_{rel})$, such that faster reactions of rS compared with the deoxyribose-containing analogs give positive values of $-\Delta\Delta G^{\ddagger}$; i.e., $-\Delta\Delta G^{\ddagger}$ is positive when the 2′-hydroxyl group speeds the reaction. Error limits of ±~0.3 kcal/mol are estimated from the range of observed values for the data for this table (not shown). [k] Values from the following paper (Herschlag et al., 1993). [l] The uncertainty is too large to determine if +1m,rS binds like +1d,rS, like rS, or with intermediate affinity.

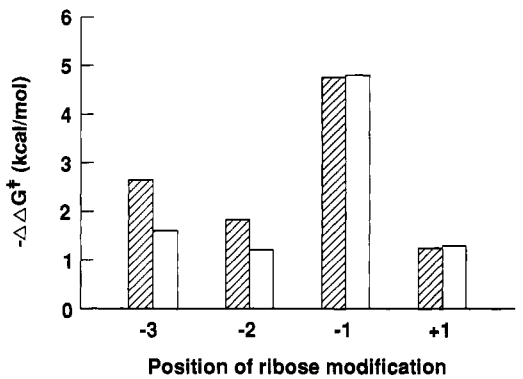


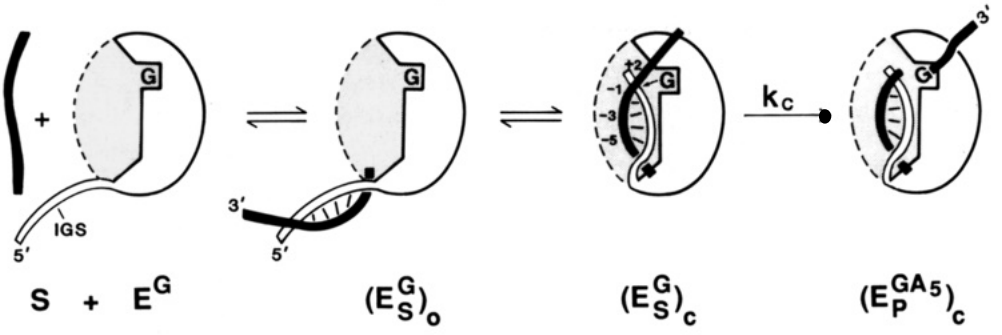
FIGURE 6: Comparison of transition-state *stabilization* from single ribose substitutions into dS (shaded) with the transition-state *destabilization* from single deoxyribose substitutions into rS (open). With this sign convention, larger values of $-\Delta\Delta G^{\ddagger}$ represent greater transition-state stabilization from the presence of the 2′-hydroxyl group for both series of oligonucleotides. Values for the dS background are from Table II and those for the rS background from Table V. "Background" refers to whether the majority of residues in the oligonucleotide are deoxyribose or ribose (see footnote 1).

DISCUSSION

The aim of this work was to understand the different binding affinities and cleavage rates of RNA and DNA substrates by the *Tetrahymena* ribozyme. The sum of the energetic effects of individual 2′-hydroxyl groups has been shown to account for close to all of the difference between rS and dS. Because the 2′-hydroxyl groups are energetically nearly independent, it has been possible to assign the contribution of individual 2′-hydroxyl groups to individual reaction steps. Finally, analysis of the effects of additional 2′-substituents has led to a crude energetic picture of this active site composed of RNA: the active site is to some extent inflexible, and it has a striking ability to discriminate between substrates at the level of a single functional group. In the following paper, the effects of 2′-substituents at the site of chemistry are analyzed, contributing to a model for interactions that stabilize the transition state (Herschlag et al., 1993).

The RNA/DNA Difference: Quantitation of Total Transition-State Stabilization. (k_{cat}/K_m) is the second-order rate constant for reaction of unbound substrate with free enzyme (or enzyme with a second substrate bound) and represents the free energy difference between the ground state of free substrate and free enzyme and the rate-limiting transition state (E + S → [E·S]‡). Thus, regardless of what interactions are present or absent in the ground-state E·S complex, comparison of (k_{cat}/K_m) values for a series of related substrates can allow identification of energetically important transition-state interactions [see Lesser et al. (1990) for a more detailed description].

The reactions of rS and dS, however, provide an example of a situation where the simple comparison of (k_{cat}/K_m) is misleading [see also Lesser et al. (1990)]. The ratio of $(k_{cat}/K_m)^S$ values [$(1.3 \times 10^8)/600$ for rS/dS; Table I] gives $\Delta\Delta G^{\ddagger}_{obsd} = 7.9$ kcal/mol, much less than the actual difference in transition-state stabilization in the transition state for the chemical step of $\Delta\Delta G^{\ddagger} = 12.2$ kcal/mol. This arises because of different rate-limiting steps for rS and dS. For rS, the binding step, rather than the chemical step, is rate-limiting, so that the difference in transition-state stabilization in the chemical step is masked. [This can be appreciated by realizing that $(k_{cat}/K_m)^S$ for rS would not increase even if the chemical step were a millionfold faster, so that stabilization of the transition state for the chemical step is not being monitored.] Thus, without an understanding of the individual steps in the ribozyme reaction, the value of $\Delta\Delta G^{\ddagger}$ would have been misleading.



	Duplex Formation	Docking	Chemical Step
Larger effects (>5 fold)	-	-3,-2	-1
Smaller effects (2-5 fold)	-6 to -1	-1,(+1)	+1

FIGURE 7: Effects on individual reaction steps in terms of the two-step binding model (Bevilacqua et al., 1992; Herschlag, 1992). According to this model, oligonucleotide is initially bound solely by base-pairing interactions in an open complex, $(E{\cdot}S)_o$; this duplex then docks into tertiary interactions to give a closed complex, $(E{\cdot}S)_c$. The numbering of positions in S is from eq 1. The effects on the "Chemical Step" have been rigorously separated from the binding effects (Table V). The apportionment of binding effects between formation of the P1 duplex ("Duplex Formation") and docking of the P1 duplex into tertiary interactions ("Docking") is based on arguments described in the text. The parentheses surrounding "+1" indicate that there is no basis for distinguishing between duplex formation and docking for the binding effect from this 2′-hydroxyl group (see text).

Furthermore, it is important to know what process is observed in the "chemical step". Previous data were consistent with the actual bond cleavage limiting the chemical step in the ribozyme reaction and revealed no requirement for any additional step, so the simplest model with rate-limiting cleavage was adopted (Herschlag & Cech, 1990a,b; Herschlag et al., 1991). However, it had not been proven for the reactions of rS and dS that the chemical step monitors the actual cleavage, rather than, for example, a conformational change of the E·G·S ternary complex. Data in the following paper provide direct evidence that the actual cleavage event is monitored in the chemical step for both rS and dS (Herschlag et al., 1993).

Do Local Interactions or Overall Helical Differences Account for the Greater Transition-State Stabilization for rS than for dS? Chimeric oligonucleotides with single ribose residues in a deoxyribose background were investigated in order to distinguish between two limiting models: (1) there are local interactions that involve specific 2′-hydroxyl groups; or (2) different *overall* duplex geometry of the DNA/RNA P1 duplex formed by dS (Figure 1) prevents the stabilization beyond base pairing that is observed with rS. The large effects of 1.3–4.8 kcal/mol from introduction of single 2′-hydroxyls at positions –3, –2, –1, and +1 (Figure 3) suggest that overall RNA/RNA helix geometry is not required for the transition-state stabilization from individual 2′-hydroxyl groups. Furthermore, the sum of the free energy effects from the individual 2′-hydroxyl groups is 13.3 kcal/mol, within error of the total difference in transition-state stabilization between rS and dS of 12.2 kcal/mol (Table II). This suggests that the effect from each 2′-hydroxyl is, to a first approximation, energetically independent of the other 2′-hydroxyl groups. If the individual 2′-hydroxyl groups at positions –3, –2, –1, and +1 were each exerting their effect by changing the overall helix geometry, then the free energies would not be additive and independent. Thus, we conclude that the majority of the effects from the 2′-hydroxyl groups arise from local interactions (model 1 above), rather than overall effects on duplex geometry (model 2 above).

The potential energetic interplay between the individual 2′-hydroxyl groups was further tested by comparing the energetic effect from addition of a single 2′-hydroxyl group to dS with the energetic effect from removal of the 2′-hydroxyl group at the same position of rS. That is, the effect of the 2′-hydroxyl group in a deoxyribose background and that in a ribose background were compared. Figure 6 shows that the effects are similar, but not identical, with significant differences of ~0.6–1.0 kcal/mol at positions –3 and –2. Thus, the energetic effects of the 2′-hydroxyl groups are not fully independent, though they are nearly independent. These deviations from complete independence may result from additional effects on the helical geometry (Chou et al., 1991; Egli et al., 1993; D.H., unpublished results).

Separation of the Effect of Each 2′-Hydroxyl into Effects on Individual Reaction Steps. The near independence described in the previous section allows a distinct, though not exact, separation of the contributions from individual 2′-hydroxyl groups in individual reaction steps. These contributions can in principle be separated into effects on each of three steps, two binding steps and the chemical step. Kinetic studies with mutant ribozymes showed that oligonucleotide binding occurs in two steps (Herschlag, 1992). Several types of data were consistent with a binding model for the mutant and wild-type ribozymes in which the oligonucleotide initially binds in an "open" complex, with only base-pairing interactions in place, followed by formation of a closed complex, with the P1 duplex docked into tertiary interactions (Figure 7). Strong support for this binding pathway for the wild-type ribozyme came from kinetic analysis of the binding of a fluorescently labeled oligonucleotide (Bevilacqua et al., 1992). In the present study, the similar values of k_{on}^S for –3d,rS and –2d,rS as for rS, despite the weaker binding of the chimeric substrates (Table V), are consistent with this model, as a rate-limiting step of duplex formation would not involve the tertiary interactions. Further evidence for this model and for its applicability in the reaction path of the wild-type ribozyme derives from studies of chimeric oligonucleotides with the wild-type and mutant ribozymes (D.H., unpublished results).

Table VI: Catalytic Effects of Specific 2′-Hydroxyl Groups on the Endonuclease Reaction

position	total effect on binding and cleavage[a]	type of catalytic effect[b]
U(−3)	12	uniform binding
C(−2)	7	uniform binding[c]
U(−1)	1900	specific effect on chemistry[d]
A(+1)	8	uniform binding and additional effect on chemistry

[a] These values are from the comparisons in Table V ($1/k_{rel}$ in the ribose background). $1/k_{rel}$ represents the hypothetical rate advantage in the reaction E + S + G → [E·S·G]‡ from addition of a 2′-hydroxyl group at the specified position to give rS (the transition state is that for the chemical step; see Table V, footnote *i*). [b] "Uniform binding" refers to the situation in which the 2′-hydroxyl group contributes the same amount in stabilizing the ground-state and transition-state complexes. A "specific effect on chemistry" refers to stabilization in the transition state that is not present in the ground state (Albery & Knowles, 1977). [c] This includes a small effect of 1.4-fold on the chemical step (Table V), so that the binding is not strictly uniform. [d] There is a small effect of ~3-fold on binding (Table V), so that the effect is not completely specific to the chemical step.

Figure 7 depicts the binding model schematically and summarizes the effect of each 2′-hydroxyl group on each reaction step. Effects on binding and chemistry were separated experimentally for the 2′-hydroxyl groups at positions −3 to +1 (Table V). The further separation of binding effects into effects on duplex formation and on docking is inferred, rather than directly measured, as described below. Table VI summarizes the effect of each 2′-hydroxyl group in terms of its effect on the reaction profile: "uniform binding" refers to the case in which the stabilization in the [E·S]‡ transition state is the same as that in the E·S ground state, and "specific effect on chemistry" refers to stabilization of the transition state that is not mirrored in the ground state (Albery & Knowles, 1977). The individual positions are discussed below, in order of 5′ to 3′ along the oligonucleotide substrate.

Positions −6 to −4 Behave as a Simple Duplex. The small enhancement in overall transition-state stabilization from 2′-hydroxyls at positions −6, −5, and −4 (Figure 2) is consistent with an effect on duplex stability. DNA/RNA duplexes tend to be less stable than RNA/RNA duplexes, though the relevant data are limited and there are exceptions for which the two duplexes have the same stability (Martin & Tinoco, 1980; Nelson & Tinoco, 1982; Walter, 1988; Bevilacqua & Turner, 1991; Hall & McLaughlin, 1991).[5] In addition, the removal of single 2′-hydroxyl groups from a model duplex that is a shortened form of the P1 duplex does indeed result in modest destabilization relative to the parent RNA/RNA duplex (Bevilacqua & Turner, 1991); the destabilization of 0.4–0.9 kcal/mol in this model duplex is similar to the transition-state stabilization of 0.5–0.8 kcal/mol from introduction of single 2′-hydroxyls at positions −6 to −4 (Table II).

The similarity of the effects of the 2′-hydroxyl and *O*-methyl substituents is consistent with a conformational origin for these effects, because both 2′-hydroxyl and *O*-methyl favor the 3′-endo sugar conformation while 2′-hydrogen (i.e., deoxyribose) favors the 2′-endo conformation (Uesugi et al., 1979; Guschlbauer & Jankowski, 1980). Recent NMR data suggest that the deoxyribose residues in DNA/RNA hybrid duplexes remain in a conformation that is similar to the 2′-endo conformation rather than converting to the 3′-endo conformation (Chou et al., 1989; Katahira et al., 1990), and the helical repeat is different for DNA/RNA duplexes than for RNA/RNA duplexes [Bhattacharyya et al., 1990; see also Gast and Hagerman (1991)]. Thus, some or all of the deoxyribose residues might need to alter their sugar conformation in order to allow the P1 duplex to dock correctly into its tertiary interactions and/or to align the reactive phosphoryl group in the active site. Finally, the absence of an inhibitory effect from the bulkier 2′-OCH_3 substituent at positions −6 to −4 provides no indication of a nearby functional group on the ribozyme (Figure 4).

In summary, limited model studies suggest that the 2′-hydroxyl groups at positions −6 to −1 should have an effect on duplex stability (Figure 7). The current study suggests that such duplex stability accounts for most of the energetic contribution of the 2′-hydroxyls at positions −6 to −4 in the ribozyme reaction; presumably, duplex stability provides a similar modest contribution at positions −3 to −1 as well. There may also be additional effects originating from differences in duplex geometry at positions −6 to −4 that are expressed in the docking or chemical steps that are not shown in Figure 7.

The 2′-Hydroxyls at U(−3) and C(−2) Are Used To Position the Substrate for Reaction. The energetic effects of the 2′-hydroxyl groups at positions −3 and −2 are large, suggesting that these groups are involved in tertiary interactions in the docked conformation (Figures 3 and 7; see also below). These effects are similar in the ground state and in the transition state (Table V and VI), suggesting that the physical interactions in the ground state are maintained in the transition state (i.e., uniform binding). The effects of the 2′-hydroxyl groups at these positions are also similar for binding of the oligonucleotide product (Bevilacqua & Turner, 1991; Pyle & Cech, 1991), further suggesting that the same physical interactions are maintained throughout the reaction. Thus, these tertiary interactions are used to hold the oligonucleotide substrate and product at the active site, positioning the reactive phosphoryl group of S for cleavage and the 3′-terminal hydroxyl group of P for nucleophilic attack in the reverse reaction.

It has been suggested that the tertiary interactions have the biological role of preventing the loss of the 5′ exon (which is analogous to P in eq 1) subsequent to the first self-splicing step, thereby promoting ligation of the exons in the second transesterification step (Herschlag & Cech, 1990a,b). According to this model, maintenance of tertiary interactions in the transition state for exon ligation (which is analogous to the reverse of the endonuclease reaction of eq 1) is necessary for splicing efficiency as it allows exon ligation to be favored over 5′ exon dissociation.[6]

The observation that the larger 2′-hydroxyl effects do not extend beyond position −3 (Figure 3) might correlate with the phylogenetic observation that the shortest P1 duplexes observed

[5] Though the sequence specificity of relative helix stability of RNA/RNA and RNA/DNA duplexes indicates that no single effect can account for all of the differences, the energy for conversion between different sugar conformations is one factor that must be considered. The trend of greater duplex stability of RNA/RNA duplexes than of RNA/DNA duplexes could arise directly from the different prefered sugar conformation for ribose and deoxyribose residues: a requirement for conversion of the deoxyribose sugar conformation in order to form the most stable hybrid duplex would partially negate the energy of duplex formation. This simple model is consistent with the limited data that suggest that oligonucleotides with 2′-substituents that favor the 3′-endo conformation tend for form stronger duplexes with RNA [see references in text and Janik et al. (1972) and Guschlbauer and Jankowski (1980)]. However, deoxyribose residues in at least some RNA/DNA hybrid duplexes are not converted to 3′-endo (Chou et al., 1989; Katahira et al., 1990). According to the simple model, these hybrid duplexes would avoid the destabilization from conversion of the sugar conformation by adopting an alternative helix geometry.

in the ~100 sequenced group I introns have three base pairs 5′ of the cleavage site (Michel & Westhof, 1990). It will be important to determine whether these tertiary interactions are conserved throughout evolution. Pyle et al. (1992) proposed an interaction between the 2′-hydroxyl group at position −3 with A302 within the intron core (see below); because A302 is highly conserved, the interaction might also be anticipated to be conserved.

Rigidity within the Active Site. Folding of RNA into a discrete and precise tertiary structure might be expected to be problematic because of a dearth of side-chain variety for tight packing of a hydrophobic core and for specifying a unique tertiary arrangement, and because of packing problems from charge repulsion of the phosphodiester backbone [e.g., Sigler (1975), Cech and Bass (1986), and Herschlag (1992)]. By these arguments, catalysis by RNA might suffer, relative to catalysis by protein, from an inability to precisely position catalytic groups within an active site. The inhibitory effect of the 2′-OCH_3 at U(−3) (Figure 4) addresses this question.

The observed ~10-fold inhibition by the 2′-OCH_3 group, relative to -H, is especially significant given its predicted stabilizing effect of ~3-fold on sugar conformation (as presumably occurs at positions −6 to −4; Figure 4 and above). The inhibition suggests that there is something nearby in the ribozyme active site. Because the inhibition is in the transition state and not just in binding, a degree of inflexibility or rigidity in the active site is indicated. The ribozyme group opposite to this *O*-methyl group cannot simply flop out of the way to avoid it; rather, there is an energetic consequence of avoiding the -OCH_3 group. The difference in transition-state stabilization of 4.3 kcal/mol between 2′-OH and -OCH_3 at U(−3) is larger than the differences of 0.6–2.1 kcal/mol obtained from this substitution at several substrate positions in the alanyl-tRNA synthetase reaction (Musier-Forsyth & Schimmel, 1992).

The Binding Site Is Specific for a Single Functional Group. The inhibition by the 2′-*O*-methyl substituent at U(−3) implies that there is an interaction of the 2′-hydroxyl group at position −3 with the ribozyme. Furthermore, this site on the ribozyme discriminates on the basis of more than simply steric bulk. Table IV shows that the site is specific relative to the smaller -H and relative to -F, -NH_2, and -NH_3^+, other groups that can participate in hydrogen-bonding interactions. Thus, this binding site composed of RNA exhibits impressive specificity on the level of a single functional group, even though RNA lacks the variety of side chains that are exploited by proteins for packing and for versatility in recognition. Similarly, there are large energetic effects from functional group substitutions on the guanosine nucleophile and from mutations within the guanosine binding site (Bass & Cech, 1984, 1986; Michel et al., 1989; Yarus et al., 1991a,b; Legault et al., 1992).

What is the nature of the binding site for the 2′-hydroxyl group at position −3? The 2′-F substituent, like the 2′-OH, favors the 3′-endo sugar conformation (Uesugi et al., 1979; Guschlbauer & Jankowski, 1980), and there is evidence that the 2′-F substituent at position −3 stabilizes duplex formation in the open complex by ~3-fold [D.H., unpublished results; see also Janik et al. (1972) and Guschlbauer et al. (1977)]. These observations render the ~100-fold preference for -OH over -F even more significant (Table IV). The inability of fluorine to substitute for the 2′-hydroxyl group is consistent with the hydroxyl group acting as a hydrogen bond donor to a functional group (or "bound" water molecule) on the ribozyme[7] and is consistent with a proposed model in which N1 of A302 (Figure 1) accepts a hydrogen bond from this 2′-hydroxyl group (Pyle et al., 1992). The ~2-fold inhibition by -F relative to -H, despite the duplex-strengthening effect of the fluorine, is also consistent with placement opposite to an electronegative hydrogen bond acceptor in the binding site.

The observed inhibition with -NH_2 is at first surprising (Table IV), since like -OH, it can both donate and accept hydrogen bonds. It is possible that a Mg^{2+} ion interacts with an oxygen lone pair of the 2′-hydroxyl group of U(−3) and that the discrimination against the -NH_2 group results from the lower affinity of Mg^{2+} for nitrogen than for oxygen. Alternatively, the inability of this functional group to replace -OH could result from differences in the number or strength, or even subtle differences in the position of these hydrogen bonding groups that perturb interactions within a highly defined binding site.

A Direct or Indirect Effect from the 2′-Hydroxyl Group at Position −2? Addition of a single 2′-OH at position −2 contributes 1.9 kcal/mol to transition-state stabilization (Figure 3). Because of the size of this effect, it is tempting to conclude that it arises, at least in part, from a direct tertiary interaction with the ribozyme. The 2′-OCH_3 substitution is less helpful than the 2′-OH (Figure 4), which could indicate that the 2′-OH serves as a hydrogen bond donor to a site on the ribozyme. However, the small rate *acceleration* from 2′-OCH_3 substitution at this position provides no indication of any nearby functional group on the ribozyme. The rate acceleration is similar to that observed at positions −6, −5, and −4, which has been attributed to effects on the P1 duplex in the absence of tertiary interactions (see above). There could nevertheless be a tertiary interaction with a hydrogen bond acceptor on the ribozyme[8] that has no significant barrier for rearrangement to accommodate the -OCH_3 group. In addition, a more complex scenario in which the 2′-*O*-methyl at position −2 coincidentally gives a net contribution to transition-state stabilization similar to that from the 2′-*O*-methyl at positions −6, −5, and −4 cannot be eliminated. For example, stabilization from the 2′-OCH_3 and 2′-OH *accepting* a hydrogen bond from the ribozyme could be offset by destabilization from a steric effect in the former case.

An alternative to the above models is that the energetic effect of this hydroxyl group arises indirectly from differences

[6] One interesting exception can occur if there is a slow conformational change before the second tranesterification reaction (e.g., bringing the 3′ splice site into the active site is slow) and the conformational change can proceed while the tertiary interactions with the 5′ exon (P) are in place. In this case, the "nonproductive" tertiary interactions can stall 5′ exon dissociation to allow time for the conformational rearrangement, even though the tertiary interaction is not present in the chemical step.

[7] It is reasonable that fluorine in a C–F bond can accept a hydrogen bond [as summarized by Withers et al. (1988)], so that an inability of fluorine to replace a hydroxyl group provides evidence against a role for the hydroxyl group as (solely) a hydrogen bond acceptor. However, the strength and even the existence of hydrogen bonds to C–F is controversial [e.g., Griffith and Roberts (1974), Curtiss et al. (1978), Murray-Rust et al. (1983), Huang and Hedberg (1989), and Takahashi et al. (1989)]. Because of this and because the analysis of hydrogen bonding equilibria in solution is complex, the best validation for the ability to use fluorine as a probe for hydrogen bond donors in binding sites appears to be the following empirical observation: glucose derivatives with -F substituents at positions 1 and 2 bind to glycogen phosphorylase more strongly than the analogous derivatives with -H and about as strongly as glucose itself, consistent with the observation of hydrogen bond donors in the active site opposite these positions in the X-ray structure of the cocrystal (Street et al., 1986).

[8] Interaction with a functional group on the ribozyme cannot be distingushed in these experiments from an interaction with a water molecule or metal ion that itself interacts with the ribozyme.

in the prefered sugar conformation. The central bases of phage 434 operator DNA have been suggested to exert an indirect effect on binding of 434 repressor and 434 Cro by altering the ability of the neighboring DNA to adopt the geometry required for binding of these proteins [Wolberger et al., 1988; Mondragon and Harrison (1991) and references therein]. An indirect effect in the ribozyme active site could arise, for example, from perturbing the position of the G·U pair at the cleavage site or from positioning 2′-OH groups of residues of the 5′ exon binding site for tertiary interactions (Figure 1). We expect that the majority of energetic effects of the order of that seen for the hydroxyl group at C(−2) will arise from direct interactions.

The 2′-Hydroxyl at Position −1 Contributes Specifically in the Transition State. In contrast to the uniform binding of the 2′-hydroxyl groups at positions −3 and −2, the large effect of the 2′-hydroxyl at position −1 is manifest almost entirely in the transition state. As described in the following paper, the stabilization from this 2′-hydroxyl group appears to result from donation of an intramolecular hydrogen bond to the incipient 3′-oxyanion in the transition state. The effect is specific to the transition state because it is not until the bond between the 3′-oxygen atom and the phosphorus atom begins to break that there is an accumulation of negative charge on the 3′-oxygen (Herschlag et al., 1993).

Effects beyond the Base-Paired Residues: Positions +1 to +3. The 2′-hydroxyl group at position +1 beyond the cleavage site contributes to transition-state stabilization, whereas the 2′-hydroxyls further from the cleavage site (positions +2 and +3) do not provide significant stabilization (Figure 3). The small effect on binding and small additional effect in the transition state from the 2′-hydroxyl group of A(+1) (Table VI and Figure 7) could arise from a direct interaction with the hydroxyl or could arise indirectly from an effect on sugar conformation. This is not clarified by the -OCH_3 substitution (Figure 4 and Table V), which gives an effect on the chemical step that is intermediate between those of the hydrogen and hydroxyl substituents. Furthermore, the oligonucleotide with the 2′-OCH_3 substituent binds with similar affinity to the oligonucleotides with the hydrogen and hydroxyl substituents.

Preferential binding to the transition state over the ground state is required for catalysis (Polyani, 1921; Pauling, 1946; Lienhard, 1973; Jencks, 1980). The additional stabilization from the A(+1) hydroxyl group in the transition state shows that the ribozyme can use functional groups away from the site of the chemical transformation in this role. This has been observed for several protein enzymes [e.g., Chipman et al. (1967), Thompson (1973, 1974), and Fierke and Jencks (1986)]. The ribozyme may take advantage of the geometrical change of the phosphoryl group at the cleavage site, going from the tetrahedral ground state to the trigonal-bipyramidal transition state, to give preferential transition-state interaction. This may be analogous to the preferential transition-state binding of the γ-phosphoryl group of ATP in the transition state for aminoacylation by tyrosyl-tRNA synthetase (Leatherbarrow et al., 1985).

A(+1), A(+2), and A(+3) are base paired in the full-length intron but not in the ribozyme reaction (Figure 1). It remains to be determined if positioning A(+1) by base pairing in the full-length P1 duplex enhances or diminishes the effect of its 2′-hydroxyl group.

Significance of the Energetic Independence. The energetic contributions of the 2′-hydroxyl groups are nearly independent, as described above. This indicates that interactions with one hydroxyl group of S are not responsible for positioning a second hydroxyl group. If this were the case, then the first 2′-hydroxyl group(s) added would contribute less observed binding energy than subsequent groups (Jencks, 1981). Thus, the observed near independence suggests that there are other interactions that position the P1 duplex (Figure 1) in its docked conformation in the transition state.[9] The nucleotides at the base of P2 and P2.1, the joining region (J1/2) that connects the P1 duplex to the rest of the intron, the conserved G·U pair at the site of reaction, the reaction-site phosphoryl group, and the leaving group oxygen atom have been implicated (Barfod & Cech, 1989; Doudna et al., 1989; Herschlag et al., 1991; Young et al., 1991; Herschlag, 1992; Piccirilli et al., 1993; W. D. Downs and T.R.C., unpublished results). In addition, there appear to be significant interactions with the ribozyme strand of P1 (i.e., the 5′ exon binding site; S. Strobel and T.R.C., unpublished results). These interactions are found on both ends of and within the P1 duplex, explaining how an oligonucleotide substrate lacking 2′-hydroxyl groups can still be positioned to facilitate cleavage.

It is informative that the 2′-*O*-methyl substituent at position −2 is not inhibitory, as this substituent is inhibitory at the adjacent −3 and −1 positions (Figure 4; Herschlag et al., 1993) and all of these 2′-substituents lie in the minor groove of the P1 duplex. This suggests that there are closer contacts or more structured sites across from positions −3 and −1. It will be interesting to uncover the structural basis for how the RNA enzyme has constructed a binding pocket for RNA and to understand how the structural features provide the energetic effects observed herein.

[9] An alternative explanation for the near-energetic independence could, in principle, be that the active site is extremely floppy. If this were the case, then an interaction of a functional group within the active site with one 2′-hydroxyl group would not help position a second functional group within the active site for interaction with, for example, a second 2′-hydroxyl group. However, the observed inhibition by the 2′-*O*-methyl substituent at U(−3) suggests that the active site is to some extent rigid (see Rigidity within the Active Site), thereby arguing against this alternative.

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REFERENCES

Albery, W. J., & Knowles, J. R. (1977) *Angew. Chem., Int. Ed. Engl. 16*, 285–293.
Barfod, E. T., & Cech, T. R. (1989) *Mol. Cell Biol. 9*, 3657–3666.
Bass, B. L., & Cech, T. R. (1984) *Nature (London) 308*, 820–826.
Bass, B. L., & Cech, T. R. (1986) *Biochemistry 25*, 4473–4478.
Bevilacqua, P. C., & Turner, D. H. (1991) *Biochemistry 30*, 10632–10640.
Bevilacqua, P. C., Kierzek, R., Johnson, K. A., & Turner, D. H. (1992) *Science 258*, 1355–1358.
Bhattacharyya, A., Murchie, A. I. H., & Lilley, D. M. J. (1990) *Nature 343*, 484–487.
Cech, T. R. (1990) *Annu. Rev. Biochem. 59*, 543–568.
Cech, T. R., & Bass, B. L. (1986) *Annu. Rev. Biochem. 55*, 599–629.
Chipman, D. M., Grisaro, V., & Sharon, N. (1967) *J. Biol. Chem. 242*, 4388–4394.

Chou, S.-H., Flynn, P., & Reid, B. (1989) *Biochemistry 28*, 2435–2443.
Chou, S.-H., Flynn, P., Wang, A., & Reid, B. (1991) *Biochemistry 30*, 5248–5257.
Couture, S. A., Ellington, D., Gerber, A. S., Cherry, J. M., Doudna, J. A., Green, R., Hanna, M., Pace, U., Rajagopal, J., & Szostak, J. W. (1990) *J. Mol. Biol. 215*, 345–358.
Curtiss, L. A., Frurip, D. J., & Blander, M. (1978) *J. Am. Chem. Soc. 100*, 79–86.
Donis-Keller, H., Maxam, A. M., & Gilbert, W. (1977) *Nucleic Acids Res. 4*, 2527–2538.
Doudna, J. A., Cormack, B. P., & Szostak, J. W. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7402–7406.
Egli, M., Usman, N., & Rich, A. (1993) *Biochemistry 32*, 3221–3237.
Fierke, C. A., & Jencks, W. P. (1986) *J. Biol. Chem. 261*, 7603–7606.
Gast, F.-U., & Hagerman, P. J. (1991) *Biochemistry 30*, 4268–4277.
Good, N. E., Winget, D., Winter, W., Connolly, T. N., Izawa, S., & Singh, R. M. M. (1966) *Biochemistry 2*, 467–477.
Griffith, R. C., & Roberts, J. D. (1974) *Tetrahedron Lett.* 3499–3502.
Guschlbauer, W., & Jankowski, K. (1980) *Nucleic Acids Res. 8*, 1421–1433.
Guschlbauer, W., Blandin, M., Drocourt, J. L., & Thang, M. N. (1977) *Nucleic Acids Res. 4*, 1933–1943.
Hall, K. B., & McLaughlin, L. W. (1991) *Biochemistry 30*, 10606–10613.
Herschlag, D. (1992) *Biochemistry 31*, 1386–1399.
Herschlag, D., & Cech, T. R. (1990a) *Biochemistry 29*, 10159–10171.
Herschlag, D., & Cech, T. R. (1990b) *Biochemistry 29*, 10172–10180.
Herschlag, D., & Cech, T. R. (1990c) *Nature (London) 344*, 405–409.
Herschlag, D., Piccirilli, J. A., & Cech, T. R. (1991) *Biochemistry 30*, 4844–4854.
Herschlag, D., Eckstein, F., & Cech, T. R. (1993) *Biochemistry* (following paper in this issue).
Huang, J., & Hedberg, K. (1989) *J. Am. Chem. Soc. 111*, 6909–6913.
Imazawa, M., & Eckstein, F. (1979) *J. Org. Chem. 44*, 2039–2041.
Janik, B., Kotick, M. P., Kreiser, T. H., Reverman, L. F., Sommer, R. G., & Wilson, D. P. (1972) *Biochem. Biophys. Res. Commun. 46*, 1153–1160.
Jencks, W. P. (1980) What Everyone Wanted to Know About Tight Binding and Enzyme Catalysis, But Never Thought of Asking, in *Molecular Biology, Biochemistry and Biophysics*, pp 3–25, Springer-Verlag, New York.
Jencks, W. P. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4046–4050.
Katahira, M., Lee, S. J., Kobayashi, Y., Sugeta, H., Kyogoku, Y., Iwai, S., Ohtsuka, E., Benevides, J. M., & Thomas, G. J., Jr. (1990) *J. Am. Chem. Soc. 112*, 4508–4512.
Latham, J. A., & Cech, T. R. (1989) *Science 245*, 276–282.
Leatherbarrow, R. J., Fersht, A. R., & Winter, G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7840–7844.
Legault, P., Herschlag, D., Celander, D. W., & Cech, T. R. (1992) *Nucleic Acids Res. 20*, 6613–6619.
Lesser, D. R., Kurpiewski, M. R., & Jen-Jacobson, L. (1990) *Science 250*, 776–786.
Lienhard, G. E. (1973) *Science 180*, 149–154.
Martin, F. H., & Tinoco, I., Jr. (1980) *Nucleic Acids Res. 8*, 2295–2299.
McConnell, T. S., Cech, T. R., & Herschlag, D. (1993) *Proc. Natl. Acad. Sci. U.S.A.* (in press).
Michel, F., & Westhof, E. (1990) *J. Mol. Biol. 216*, 585–610.
Michel, F., Hanna, M., Green, R., Bartel, D. P., & Szostak, J. W. (1989) *Nature 342*, 391–395.
Mondragon, A., & Harrison, S. C. (1991) *J. Mol. Biol. 219*, 321–334.
Murray-Rust, P., Stallings, W. C., Monti, C. T., Preston, R. K., & Glusker, J. P. (1983) *J. Am. Chem. Soc. 105*, 3206–3214.
Musier-Forsyth, K., & Schimmel, P. (1992) *Nature 357*, 513–515.
Nelson, J. W., & Tinoco, I., Jr. (1982) *Biochemistry 21*, 5289–5295.
Pauling, L. (1946) *Chem. Eng. News 24*, 1375–1377.
Piccirilli, J. A., Vyle, J. S., Caruthers, M. H., & Cech, T. R. (1993) *Nature 361*, 85–88.
Polyani, M. (1921) *Z. Elektrochem. 27*, 142–150.
Pyle, A. M., & Cech, T. R. (1991) *Nature (London) 350*, 628–631.
Pyle, A. M., McSwiggen, J. A., & Cech, T. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8187–8191.
Pyle, A. M., Murphy, F. L., & Cech, T. R. (1992) *Nature 358*, 123–128.
Robertson, D. L., & Joyce, G. F. (1990) *Nature 344*, 467–468.
Rose, I. A., O'Connell, E. L., Litwin, S., & Bar Tana, J. (1974) *J. Biol. Chem. 249*, 5163–5168.
Sigler, P. B. (1975) *Annu. Rev. Biophys. Bioeng. 4*, 447–527.
Street, I. P., Armstrong, C. R., & Withers, S. G. (1986) *Biochemistry 25*, 6021–6027.
Sugimoto, N., Sasaki, M., Kierzek, R., & Turner, D. H. (1989) *Chem. Lett.*, 2223–2226.
Takahashi, L. H., Radhakrishnan, R., Rosenfield, R. E., Jr., Meyer, E. F., Jr., & Trainor, D. A. (1989) *J. Am. Chem. Soc. 111*, 3368–3374.
Thompson, R. C. (1973) *Biochemistry 12*, 47–51.
Thompson, R. C. (1973) *Biochemistry 13*, 5495–5501.
Uesugi, S., Miki, H., Ikehara, M., Iwahashi, H., & Kyogoku, Y. (1979) *Tetrahedron Lett. 42*, 4073–4076.
Walker, G. T. (1988) *Nucleic Acids Res. 16*, 3091–3099.
Williams, D. M., Benseler, F., & Eckstein, F. (1991) *Biochemistry 30*, 4001–4009.
Withers, S. G., Street, I. P., & Percival, M. D. (1988) Fluorinated Carbohydrates as Probes of Enzyme Specificity and Mechanism, in *Fluorinated Carbohydrates. Chemical and Biochemical Aspects*, pp 59–77, American Chemical Society, Washington, DC.
Wolberger, C., Dong, Y., Ptashne, M., & Harrison, S. C. (1988) *Nature 335*, 789–795.
Yarus, M., Illangesekare, M., & Christian, E. (1991a) *Nucleic Acids Res. 19*, 1297–1304.
Yarus, M., Illangesekare, M., & Christian, E. (1991b) *J. Mol. Biol. 222*, 995–1012.
Young, B., Herschlag, D., & Cech, T. R. (1991) *Cell 67*, 1007–1019.
Zaug, A. J., Been, M. D., & Cech, T. R. (1986) *Nature 324*, 429–433.
Zaug, A. J., Grosshans, C. A., & Cech, T. R. (1988) *Biochemistry 27*, 8924–8931.