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The Importance of Being Ribose at the Cleavage Site in the *Tetrahymena* Ribozyme Reaction†

Daniel Herschlag,*,‡ Fritz Eckstein,§ and Thomas R. Cech‖

Department of Biochemistry, B400 Beckman Center, Stanford University, Stanford, California 94305-5307, Howard Hughes Medical Institute, Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215, and Max-Planck-Institut fuer experimentelle Medizin, Hermann-Rein Strasse 3, W-3400 Gottingen, Germany

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ABSTRACT: The ribozyme derived from the intron of *Tetrahymena thermophila* pre-rRNA catalyzes a site-specific endonuclease reaction with both RNA and DNA oligonucleotides. The total transition-state stabilization by the ribozyme, encompassing the binding and chemical steps, is 4.8 kcal/mol greater with a single ribose at the cleavage site relative to the all-deoxyribose substrate. Here we show that this effect is specific to the chemical transition state, with a contribution of only ~0.7 kcal/mol toward binding. Substrates with a series of 2′-substituents, -OH(ribo), -F_2 (2′,2′-difluoro-2′-deoxyribo), -F(2′-fluoro-2′-deoxyribo), and -H(deoxyribo), follow a linear free energy relationship between the rate of the chemical step of the ribozyme-catalyzed reaction and the pK_a of the leaving group, with slope $\beta_{leaving\ group} \approx -0.8$. Because proton donation to the 3′-oxygen atom from a general acid of the ribozyme would be expected to render the rate insensitive to the pK_a of the leaving group, it is suggested that this ribozyme does not employ general acid catalysis. The 2′-OCH_3 (2′-methoxy-2′-deoxyribo) substituent does not follow this correlation, apparently due to steric hindrance within the active site. The rate of cleavage of the 2′-substituted substrates by the ribozyme follows the order 2′-F_2 > -F > -H, suggestive of an inductive effect, i.e., acceleration of the reaction by electron-withdrawing groups. The 2′-OH group provides the largest transition-state stabilization. Because of uncertainty in the relative effect of the 2′-OH and 2′-H substituents on the pK_a of the neighboring 3′-oxygen leaving group, we do not discount the possibility of interactions between the 2′-hydroxyl group and the ribozyme that further enhance reactivity. Nevertheless, the 2′-OH effect can be explained at least partially by an intramolecular hydrogen bond to an incipient oxyanion at the neighboring 3′-position. This oxyanion is forming as the phosphodiester bond is breaking, explaining why the stabilization is specific to the transition state. Analogous differential hydrogen bonding might be widely used by enzymes to achieve selective transition-state stabilization.

Several distinct classes of RNA enzymes or "ribozymes" have been discovered, all catalyzing phosphoryl-transfer reactions (Kruger et al., 1982; Guerrier-Takada et al., 1983; Buzayan et al., 1986; Peebles et al., 1986; Prody et al., 1986; Schmelzer & Schweyen, 1986; Van der Veen et al., 1986; Forster & Symons, 1987; Kuo et al., 1988; Hampel & Tritz, 1989; Saville & Collins, 1990). More recently, the ribozyme from *Tetrahymena thermophila* pre-rRNA, which normally catalyzes an RNA self-splicing event, and a ribosome, stripped of nearly all of its protein components, have been shown to catalyze reactions involving a carbon ester *in vitro* (Noller et al., 1992; Piccirilli et al., 1992). Indeed, it has even been suggested that RNA was a versatile catalyst in a metabolically complex "RNA world" [e.g., Benner et al. (1989)].

The *Tetrahymena* ribozyme provides an estimated rate enhancement of ~10^{11}-fold over the uncatalyzed reaction, comparable to the rate enhancement observed for many protein enzymes (Herschlag & Cech, 1990a). But we are just beginning to understand *how* RNA can provide efficient catalysis (Cech et al., 1992). Can RNA, for example, efficiently utilize general acid/base catalysis? Proteins contain imidazole side chains on histidine residues with pK_a values near neutrality, optimally suited for general acid/base catalysis under physiological conditions. RNA lacks such a group, instead having functional groups with pK_a values above ~9 or less than ~4 (Saenger, 1983). RNA might get by with less efficient general acid/base catalysis, utilizing functional groups with pK_a values away from neutrality. Alternatively, if RNA uses general acid/base catalysis, the folded RNA structure might provide an environment that perturbs pK_a values toward neutrality, as occurs in protein active sites [e.g., Schmidt and Westheimer (1971)].

Single-stranded DNA can be a substrate for the *Tetrahymena* ribozyme, though it is a much worse substrate that RNA (Herschlag & Cech, 1990c; Robertson & Joyce, 1990). RNase P and group II introns also cleave at deoxyribose residues with reduced efficiency (Forster & Altman, 1990; Morl et al., 1992). Is there some incompatibility with deoxyribose that prevents it from binding properly or interacting properly with these catalytic centers, or is the lower reactivity of DNA what one would expect from simple chemical considerations?

In this paper we have systematically varied the 2′-substituent at the cleavage site of the oligonucleotide substrate, following the approach of physical organic chemistry, in order to learn about the nature of the transition state for the chemical step of the *Tetrahymena* ribozyme (Jencks, 1969; Lowry & Richardson, 1981). Replacement of the ribose residue at the cleavage site by a deoxyribose residue causes only a small

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* To whom correspondence should be addressed.
‡ Stanford University.
§ Max-Planck-Institut fuer experimentelle Medizin.
‖ University of Colorado.

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Table I: Rate Effects from 2′-Substituents at the Cleavage Site of the DNA Substrate[a]

2′-substituent at U(−1)	$(k_{cat}/K_m)^S$ (M^{-1} min^{-1})	k_{rel}[b]
-H	400	(1)
-F	9600	24
-F_2[c]	15 000	37
-OH	680 000	1700
-OCH_3	<20	<0.05
		~0.005[d]

[a] Substrates consisted of all-deoxyribose residues (i.e., the deoxyribose background) except at U(−1), which contained the specified 2′-substituent (e.g., -F = −1F,dS; see footnote 1). Single-turnover reactions with 200–1000 nM ribozyme, 2 mM G, and ~5 nM 5′-end-labeled S in 50 mM sodium MES, pH 7, and 10 mM $MgCl_2$ at 50 °C. The observed rate constants increased linearly with ribozyme concentration, indicating that $(k_{cat}/K_m)^S$ was followed. G was shown to be near saturating for dS with K_m^G = 1 mM (Herschlag & Cech, 1990c; McConnell et al., in press; data not shown), and no correction to saturation was made. A similar dependence on G concentration was obtained with −1r,dS (McConnell et al., in press; and data not shown). [b] $k_{rel} = (k_{cat}/K_m)^S/(k_{cat}/K_m)^{dS}$ represents the rate constant for cleavage of the chimeric substrate relative to that of dS. Values are from side-by-side comparisons so that the k_{rel} values are more accurate than the individual values of $(k_{cat}/K_m)^S$. [c] 2′,2′-Difluoro-2′ deoxyribo*thymidine*, was used rather than 2′-deoxyuridine as with the other substituents. Control experiments comparing the reactivity of dS with dU vs dT at position −1 revealed no significant rate effect from the 5′-methyl group of T (rate effect <30%; data not shown). [d] From comparison of −1m,−3r,dS with −3r,dS. Addition of the ribose moiety at position −3 speeds the reaction of dS 65-fold, thereby allowing reaction with a 2′-OCH_3 group at position −1 to be detected. Nevertheless, there is about 3-fold more cleavage at position −3 than at the normal cleavage site (−1) with −1m,−3r,dS (data not shown; see Results). The value reported in the table represents only the reaction to give the normal product, P. It was determined from the observed rate constant for cleavage and the fraction of the total product corresponding to P. The same value was obtained from the initial rate of formation of P (data not shown). All of the other substrates gave exclusively the normal product.

decrease in binding affinity [consistent with previous work by Bevilacqua and Turner (1991) and Pyle and Cech (1991)]. In contrast, there is a large reduction in the cleavage rate with a deoxyribose sugar at the cleavage site, and this reduction is close to that expected from the less stable leaving group (the 2′-deoxyribose 3′-oxyanion). Because protonation of the 3′-oxygen atom in the transition state would be expected to render the rate less sensitive to the stability of the leaving group, our results further suggest that the *Tetrahymena* ribozyme does not employ a general acid catalyst. Rather, a metal ion appears to be used to stabilize the developing negative charge on the leaving group oxyanion (Piccirilli et al., 1993).

MATERIALS AND METHODS

All of the materials and methods were the same as or analogous to those described in the preceding paper (Herschlag et al., 1993), with the exception of oligonucleotides containing 2′,2′-difluoro-2′-deoxyribothymidine. These oligonucleotides were synthesized from the 3′-phosphoramidite of 5′-(dimethoxytrityl)-2′,2′-difluoro-2′-deoxyribothymidine (Hertel et al., 1988; Richardson et al., 1992), which was a generous gift from L. Hertel (Lilley Research Laboratories, Eli Lilley & Co.).

RESULTS

Reactions in the Deoxyribose Background. The effects of 2′-substituents on the pentose sugar at the cleavage site were investigated to provide insight into interactions that are important in stabilizing the transition state. Table I shows the absolute and relative values of $(k_{cat}/K_m)^S$ for a series of

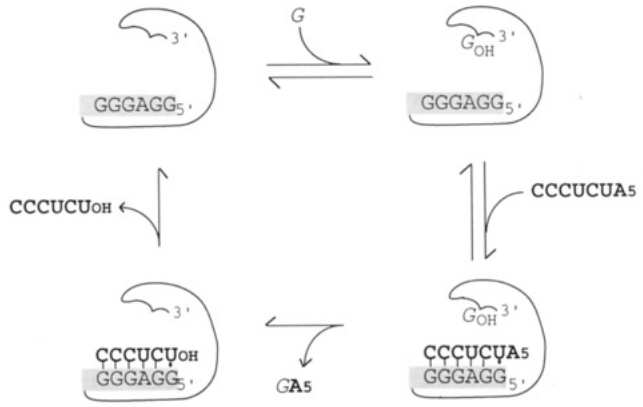

FIGURE 1: Site-specific endonuclease reaction catalyzed by the *Tetrahymena* ribozyme. The oligonucleotide substrate (S, CCCUCUAAAAA) and product (P, CCCUCU) are base paired to the "internal guide sequence" (shaded) of the ribozyme to form the P1 duplex. The reaction is analogous to the first step of the self-splicing reaction (Cech & Bass, 1986; Cech, 1990). The L-21 *Sca*I ribozyme used in these experiments is missing 21 nucleotides from the 5′ end and 5 nucleotides from the 3′ end of the full-length intron (Zaug et al., 1988).

oligonucleotide substrates with a deoxyribose "background", i.e., having deoxyribose residues at all positions other than U(−1) (Figure 1).[1] As described in the preceding paper, $(k_{cat}/K_m)^S$ represents the reaction $E{\cdot}G + S \rightarrow [E{\cdot}G{\cdot}S]^{\ddagger} \rightarrow$ products. Thus, differences in k_{rel} (Table I) reflect effects on the binding equilibrium as well as effects on the chemical step [see Herschlag et al. (1993)]. The effects are quite large, spanning a range of reactivities of $>10^5$, which represents differences in overall transition-state stabilization of 8 kcal/mol.[2]

Reactions in the Ribose Background. The effects of these substituents on the binding step and on the chemical step were separated in experiments with oligonucleotide substrates in the ribose background[1] (Table II). The stronger binding in the ribose background than in the deoxyribose background facilitated these determinations (Herschlag & Cech, 1990c; Herschlag et al., 1993). There are small but significant effects of the 2′-substituents on binding [Table II, $1/(K_d^S)_{rel}$]. Similar small effects from the 2′-OH and -H at U(−1) were observed for the binding of CCCUCU and CUCU (Bevilacqua & Turner, 1991; Pyle & Cech, 1991). However, the vast majority of the effect is exerted in the chemical step [$(k_{cat}/K_m)^G_{rel}$; see below].

[1] Oligonucleotides are abbreviated: 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 residues, respectively. Chimeric oligonucleotides are named by specifying residues that are different from the ribose "background" of rS or from the deoxyribose "background" of dS. For example, −1r,dS signifies a ribose residue at position −1 from the cleavage site in the deoxyribose background, i.e., with deoxyribose residues at all other positions: dCdCdCdUdCrUdAdAdAdAdA); likewise, −1d,rS signifies a deoxyribose residue at position −1 with ribose residues at all other positions: rCrCrCrUrCdUrArArArArA. Other sugar derivatives are signified as follows: m, 2′-methoxy-2′-deoxyribose; F, 2′-fluoro-2′-deoxyribose; F_2, 2′,2′-difluoro-2′-dideoxyribose. The following abbreviations are also used: E, ribozyme; G, guanosine; P′, CCCUC; P″, CCCU; 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.

[2] Values of $\Delta\Delta G^{\ddagger}$ are calculated from the equation: $\Delta\Delta G^{\ddagger} = -RT \ln k_{rel}$. This is derived from $\Delta G^{\ddagger} = -RT \ln(kh/k_BT)$ with k in units of s^{-1}, where R = 1.987 cal mol^{-1} K^{-1}, T = 323 K (50 °C), $h = 1.58 \times 10^{-34}$ cal s, and $k_B = 3.30 \times 10^{-24}$ cal K^{-1}.

Table II: Effect of Individual 2′-Substituents at U(−1) of the RNA Oligonucleotide Substrate on the Binding Step and on the Chemical Step[a]

substituent	k_{on}^{S} [b] (10^8 M^{-1} min^{-1})	k_{off}^{S} [c] (min^{-1})	K_d^S [d] (nM)	$1/(K_d^S)_{rel}$ [e]	$k_c(-G)$ [f,g] (min^{-1})	$(k_{cat}/K_m)^G$ [g,h] (M^{-1} min^{-1})	$(k_{cat}/K_m)^G_{rel}$ [e]
-H	~1.4[i]	1.0 ± 0.3	7	(1)	~4×10^{-4}	15	(1)
-F	1.6	1.0 ± 0.4	6	1	0.02	1000	70
-F_2	1.6	2.5 ± 0.5	20[j]	0.4	0.03	4600	310
-OH	1.5	0.35 ± 0.15	2.3	3	0.11	8800	590

[a] Determinations were made at 50 °C in 10 mM $MgCl_2$ and 50 mM sodium MES at pH 5.2 or 7.0, as specified. All reactions were performed side-by-side with reactions of rS ("-OH"), so 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 2–10 nM ribozyme, 1 nM 5′-end-labeled S, and 2 mM G at pH 7. The observed rate constants for the first-order disappearance of S increased linearly with ribozyme concentration, and G was shown to be saturating, indicating that $(k_{cat}/K_m)^S$ was followed. Pulse–chase experiments showed that binding rather than a subsequent step is rate limiting for all of the substrates except for −1d,rS (see footnote *i*) so that $(k_{cat}/K_m)^S = k_{on}^S$, the rate constant for binding of S to the ribozyme, 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), as described in the preceding paper (Herschlag et al., 1993). For all of the substrates except −1d,rS the chemical step with saturating G (≥1 mM) is much faster than the dissociation of S (k_{off}^S) so that the amount of 5′-end-labeled P formed identifies the amount of S that had bound (productively) prior to the "chase"; in all cases the extent of reaction in this control was >85%. This allowed determination of k_{off}^S by two independent calculations, based on the fraction of labeled S trapped as P and based on the observed rate constant for formation of labeled P, as described previously (Herschlag & Cech, 1990a; Herschlag et al., 1993). There was good agreement between the approaches, as demonstrated by the error limits, which are based on the range of calculated values. For −1d,rS, the amount of S that had bound (productively) prior to the "chase" could not be determined in the same manner, so k_{off}^S was calculated from the observed rate constant for formation of P. The values of k_{off}^S obtained agreed with those calculated *assuming* a similar amount of bound S as in pulse–chase experiments with the other oligonucleotide substrates. A second pulse–chase method gave the same value for k_{off}^S for −1d,rS. In this method, subsequent to formation of the E·S* complex (during t_1), unlabeled oligonucleotide competitor was added at $t_2 = 0$ and left for a variable time prior to addition of a saturating concentration of G at $t_3 = 0$. The reaction of G with 5′-end-labeled S that had not dissociated from the ribozyme (E·S*) proceded during t_3, and the reaction was quenched. (Control experiments established the time t_3 required for the reaction of the bound 5′-end-labeled S to be completed; as noted above, not all of the bound species reacts, but this does not affect the determination of k_{off}^S.) The value of k_{off}^S was obtained from the amount of 5′-end-labeled product formed as a function of time t_2 (data not shown). [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, it has been 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] Relative to the value for −1d,rS ("-H"). Values of $1/(K_d^S)_{rel}$ and $(k_{cat}/K_m)^G_{rel}$ larger than 1 represent stronger binding and faster reaction. [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 5.2, with saturating ribozyme (see footnote *g*). [g] For most experiments 200 nM ribozyme was used, which is saturating for all of the oligonucleotides (based on the K_d^S values in the table and control experiments in which the ribozyme concentration was varied without affecting the rate of reaction; data not shown). [h] Second-order rate constant for attack by G in the single-turnover reaction: E·S + G → products in sodium MES, pH 5.2, with saturating ribozyme (see footnote *g*). [i] For −1d,rS the rate constant for the chemical step is similar to k_{off}^S so that $(k_{cat}/K_m)^S$ is partially limited by both the binding and chemical steps [see Herschlag and Cech (1990a,b) for a more detailed explanation]. The value of $k_{on}^{-1d,rS}$ was therefore obtained by calculation and is less certain than the other values. [j] Determination by a second method gave a value in reasonable agreement. The single-turnover reaction with [E] >> [S] and 0.5 μM G was followed as a function of [E]. A value of $k_{off}^S = 4$ min^{-1} was determined from the observed value of $K_m^E = 30$ nM according to the equation: $K_m^E = (k_{off}^S + k_{cat})/k_{on}^S$, with $k_{on}^S = 1.6 \times 10^8$ M^{-1} and $k_{cat} = 0.5$ min^{-1}.

The cleavage reactions in the ribose background were followed at pH 5 rather than pH 7 (Table II) because there is evidence that the same step is rate-limiting for substrates in the ribose background at pH 5 as in the deoxyribose background at pH 7, whereas a different step may be rate-limiting for reactions in the ribose background at pH ≥7. Figure 2 shows the pH dependence for cleavage of dS, −3r,dS, and −1r,dS. [This dependence was also observed for dS, −1F,dS, and −1r,dS from pH 6.1 to 7.9 in analogous experiments performed at 30 °C (data not shown)]. The observation of the same pH dependence for these substrates suggests that the same step is rate-limiting for the different substrates and also argues against the unlikely scenario in which a proton is lost from the 2′-hydroxyl group. For the substrates in the ribose background (Table II), the pH dependence of $(k_{cat}/K_m)^G$ was the same as that obtained in Figure 2 at low pH, but not at the higher pH values; the rate constant for reaction of rS leveled off at pH ≥ 7 (D.H., unpublished results; the origin of the pH dependence for rS, which may reflect a change in the rate-limiting step, is currently under investigation). Thus, data for the substrates in the ribose background obtained at the low pH (5) have been used in the analysis herein.[3]

The second-order rate constant $(k_{cat}/K_m)^G$ (Table II) represents the reaction E·S + G → [E·S·G]‡, so the 2′-substituents could affect this rate constant through an effect on binding of G or on reactivity of the E·S·G ternary complex. However, the value of K_m^G was affected only slightly, if at all, in single-turnover reactions of E·S (with varying [G]) for the oligonucleotide substrates investigated in Table II. Values of K_m^G were determined at low temperature and low pH to slow the single-turnover reaction of E·S (50 mM sodium MES, pH 5.2, 200 nM ribozyme, ~1 nM 5′-end-labeled S, and 0–2 mM G at 30 °C; data not shown). These conditions are expected to render $K_m^G = K_d^G$, the equilibrium constant for dissociation of G from E·S·G. This is because E·S is formed rapidly, so that binding of S is not rate-limiting, and the reaction is slow, so that G can equilibrate between bound and free prior to reaction of the ternary E·S·G complex (Herschlag & Cech, 1990c; Herschlag et al., 1991; McConnell et al., in press). Thus, we conclude that essentially all of the difference in $(k_{cat}/K_m)^G$ values arises from differences in the rate constant for the chemical step, not from differences in the affinity for G. The pH dependence and the linear free energy relationship described below also suggest that the actual chemical cleavage step is rate-limiting, rather than a conformational change of the E·S·G complex (see Discussion). Thus, the differences in the values of $(k_{cat}/K_m)^G_{rel}$ (Table II) are attributed to differences in the actual chemical cleavage, rather than differences in the affinity for G or in a conformational change of the E·S·G ternary complex.

The Behavior of the 2′-Fluoro and 2′-Difluoro Substituents. The effects of the 2′-H, -OH, -OCH_3, and -F substituents on helix stability and sugar conformation have been investigated in a few instances [see references in Herschlag et al. (1993)]. In contrast, the only information of this type regarding the

[3] The binding data in Table II were obtained at pH 7. Experiments with rS at pH 5 and 7 showed that K_d^S is not significantly affected by this difference in pH (data not shown). For the cleavage reaction, use of data in the ribose background obtained at pH 7 would result in quantitative differences, but would not change any of the conclusions herein.

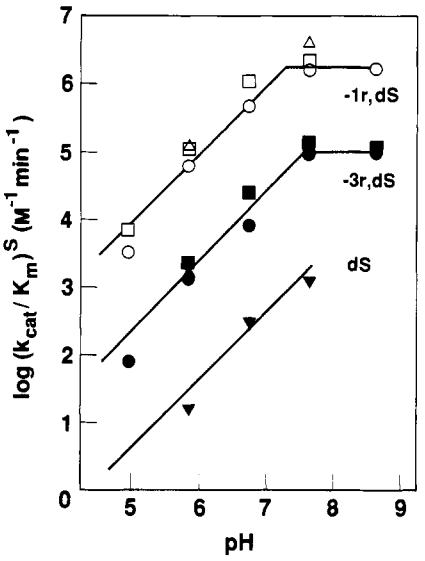

FIGURE 2: Effect of pH on ribozyme-catalyzed cleavage of oligonucleotide substrates. Values of $(k_{cat}/K_m)^S$ for -1r,dS (open symbols), -3r,dS (closed symbols), and dS (inverted triangles). Different symbols for -1r,dS and -3r,dS represent independent experiments. The lines have slopes of 1 from pH 5 to ~7.5 The reactions of -1r,dS and -3r,dS were carried out with 100–300 nM ribozyme, 2 mM G, and ~5 nM 5′-end-labeled S. That with dS was carried out with 2 μM ribozyme and 0.8 mM G. [No correction to saturating G was made; K_m^G = 1 mM (Herschlag & Cech, 1990c; McConnell et al., in press)]. The value of k_{obsd} was shown to be linear in ribozyme concentration at pH 6.7 for each substrate so that $(k_{cat}/K_m)^S$ is being followed. 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; initial experiments with rS and -1d,rS support this assumption (D.H., unpublished results). 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 no attempt was made herein to control for potential salt- and buffer-specific effects. However, initial results from a detailed analysis of the pH dependence of individual reaction steps suggest that salt effects and effects on the binding of G or S cannot account for the slope of 1 in the pH dependencies (McConnell et al., in press; D. Knitt and D.H., unpublished results). Thus, the pH dependence that is suggested from this figure is interpreted in the Discussion.

2′-F_2 substituent of which we are aware is that this substituent destabilizes DNA·DNA duplexes (Richardson et al., 1992). We therefore probed its effect at position –3 of the ribozyme substrate (Figure 1). This position was chosen because only the thymine derivative was available, and U is the base at this position of the substrate. In the deoxyribose background, the 2′-F_2 substituent at position –3 (i.e., –3F_2,dS) slowed the reaction >5-fold relative to the reaction of dS at 50 °C (E·G + S → [E·G·S]‡; conditions as in Table I; data not shown). Analogous experiments at 30 °C allowed greater sensitivity and revealed an inhibitory effect of ~10-fold from this substituent (data not shown).

Because thymine was the base with the 2′-F_2 nucleotide, whereas uracil was the base with all of the other 2′-substituent analogs, the reactivities of substrates containing dT and dU were compared. Within experimental uncertainty, dT and dU gave the same reactivity at position –3 and at position –1 (in the deoxyribose background with conditions as in Table I, performed at both 50 and 30 °C; data not shown). Thus, the reactions of substrates with thymine and uracil bases are directly comparable. The equivalence of dT and dU also suggests that the 5′-positions of the uracils at position –3 and position –1, situated in the major groove of the P1 duplex (Figure 1), are not in contact with the ribozyme.

Substitution of 2′-F_2 at position –1 resulted in a large increase in the rate of cleavage relative to 2′-H, in contrast to the inhibitory effect from this substitution at position –3 (above and Table I). 2′-F also increased the rate of cleavage when substituted at position –1 and decreased cleavage when substituted at position –3 (Table I; Herschlag et al., 1993). The inhibitory effects at U(–3) of 2′-F_2 and 2′-F are consistent with proximity of a hydrogen bond acceptor in the active site of the ribozyme, as described in the previous paper. The ~5-fold larger inhibitory effect of 2′-F_2 than -F (E·G + S → [E·G·S]‡; reactions at 30 °C; data not shown) is consistent with an unfavorable conformational effect from introduction of the second fluorine atom. The stimulatory effects of these substituents at the site of bond cleavage are attributed to inductive effects, as described in the Discussion.

The Unusual Behavior of the 2′-Methoxy Substituent. In contrast to the rate enhancement from the 2′-F and -F_2 substituents at U(–1), the 2′-methoxy substituent slowed the reaction relative to that with the 2′-H substituent. Indeed, no reaction was observable in the deoxyribose background (Table I). A ribose moiety was therefore added at position –3, as this substitution speeds cleavage at U(–1) (Herschlag et al., 1993). Comparison of the reactions of –3r,dS and –1m,–3r,dS allowed determination of the effect of the 2′-methoxy group on the rate constant for formation of P: k_{rel}(-OCH_3/-H) ≈ 0.005 (Table I).

In addition to slowing the reaction, the *O*-methyl substituent at U(–1) caused miscleavage. For –1m,–3r,dS the primary cleavage occurred two residues 5′ of the normal site to give CCCU (P″). Approximately 80% of the product was P″ rather than P (CCCUCU; data not shown). (The rate constant in Table I represents only the cleavage at the correct position to form P.) Abberrant cleavage of the substrate was even more pronounced in the ribose background: with –1m,rS, only ~2% of the product was P, the remainder consisting of P″ and P′ (CCCUC) in a 3:1 (P″:P′) ratio (data not shown). Greater miscleavage in the ribose background is expected, because cleavage is enhanced by 2′-hydroxyl groups one and two positions preceding a cleavage site (Herschlag, 1992; see below). The 2′-*O*-methyl substituent slowed the formation of P in the ribose background, as it did in the deoxyribose background: cleavage of –1m,rS in the E·S complex to give P was slowed ~5000-fold upon replacement of the 2′-OH at U(–1) by -OCH_3; the cleavage was even slower than that with 2′-H (data not shown).

The 2′-*O*-methyl substituent had an additional effect, destabilizing binding of S by ~50-fold. The dependence of the rate of cleavage on the concentration of ribozyme in single-turnover reactions gave K_m^E = 100 nM (Figure 3). Under the conditions of this experiment, the following relationship is expected: $K_m^E = (k_{cat} + k_{off}^S)/k_{on}^S$, where k_{cat} is the observed rate constant for cleavage with saturating ribozyme. The observed value of $(k_{cat}/K_m)^{-1m,rS} = 2 \times 10^7$ M^{-1} min^{-1} obtained with 2 mM G and 10–100 nM ribozyme (data not shown) sets a minimum value for $k_{on}^{-1m,rS}$. This minimum value of $k_{on}^{-1m,rS}$ = 2×10^7 M^{-1} min^{-1} and k_{cat} = 0.35 min^{-1} (for cleavage to form all products; Figure 3) gives the value of $K_d^{-1m,rS}$ = k_{off}^S/k_{on}^S = 80 nM, a minimum estimate for $K_d^{-1m,rS}$. A somewhat larger value for $k_{on}^{-1m,rS}$ of ~10^8 M^{-1} min^{-1} is suggested by the observation that a number of oligonucleotides have this value of k_{on}^S for binding to the wild-type and mutant ribozymes (Table II; Young et al., 1991; Herschlag et al., 1993; T. McConnell, T.R.C., and D.H., unpublished results). This larger estimate for $k_{on}^{-1m,rS}$ gives $K_d^{-1m,rS} \approx K_m^E$ = 100 nM according to the above equations. This value of $K_d^{-1m,rS}$

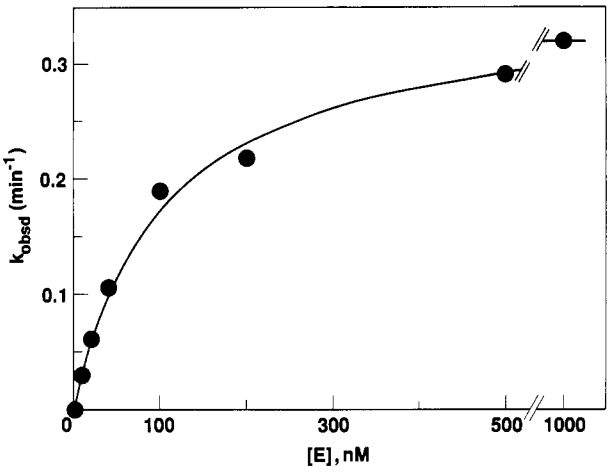

FIGURE 3: Determination of $K_d^{-1m,rS}$ from the dependence of the rate of cleavage on ribozyme concentration. Single-turnover reactions were carried out with 0.1 mM G, ~2 nM 5'-end-labeled -1m,rS, 50 mM sodium MES, pH 7, and 10 mM $MgCl_2$ at 50 °C. The line is a theoretical fit to a hyperbolic dependence on ribozyme concentration: $k_{obsd} = k_{cat}[E]/(K_m^E + [E])$, with K_m^E = 100 nM and k_{cat} = 0.35 min^{-1} (for cleavage to form both the correct and miscleaved products; see Results). Evidence that the value of K_m^E is equal to $K_d^{-1m,rS}$ is described in the text.

is ~50-fold larger than the dissociation constant for the all-ribose substrate (Table II).

Destabilization of binding of S can account for the abberrant cleavage when 2'-*O*-methyl is substituted at position -1. Abberrant cleavage to give shorter products, such as P' and P'', was previously shown to result from docking of the P1 duplex (Figure 1) into active site tertiary interactions in alternative registers (Herschlag, 1992). In the alternative registers the wrong phosphodiester bond is juxtaposed to the nucleophilic guanosine, leading to formation of the abberrant products. It was concluded that in several mutant ribozymes docking into alternative registers is favored, relative to the wild-type ribozyme, thereby facilitating the abberrant cleavage (Herschlag, 1992).

The weaker binding of -1m,rS suggests that the 2'-methoxy group at U(-1) destabilizes docking in the normal register. Abberrant cleavage could then be enhanced because the fraction of bound substrate docked into the alternative registers is greater for -1m,rS than for rS. There are no data to support the alternative possibility that binding in the alternative registers is stabilized by the 2'-methoxy group at U(-1).

Even though there is only a modest 5-fold coupling between the binding of rS and G (McConnell et al., in press), the 2'-methoxy moiety of U(-1) in -1m,rS might still have disrupted binding of G. However, a control experiment carried out under reaction conditions that are thought to give $K_m^G = K_d^G$ [see McConnell et al. (in press)] showed no large effect of bound -1m,rS on K_m^G. (Conditions: 100 nM ribozyme and ~5 nM 5'-end-labeled -1m,rS at 30 °C, to give complete formation of the E·S complex, 0–2.0 mM G, 50 mM sodium MES, pH 7, and 10 mM $MgCl_2$; K_m^G was compared to the values obtained for the cleavage of -1r,dS and dS with saturating and subsaturating concentrations of ribozyme.) In addition, there was no significant change in the ratio of P'': P':P as the concentration of G was changed (data not shown). It remains possible that the 2'-methoxy group slows the reaction of bound substrate by disrupting the alignment of G in the transition state, but not in the ground state.

A Linear Free Energy Relationship. As the 2'-substituent of U(-1) is varied, both the rate of the chemical step and the estimated pK_a of the 3'-hydroxyl of the leaving group change

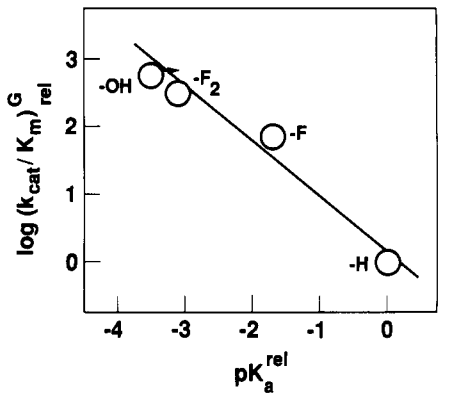

FIGURE 4: Linear free energy relationship for the ribozyme reaction. This plot shows the dependence of the rate of cleavage on the pK_a of the sugar at position -1. The line has a slope of $\beta_{lg} = \delta (\log k)/\delta pK_{leaving\ group} = -0.8$. The values of $(k_{cat}/K_m)^G$ are from Table II. The values of pK_a^{rel} are the pK_a of the leaving group 3'-hydroxyl group minus that for deoxyribose (2'-H). They were *estimated* as follows. The values for -F and -F_2 derivatives are taken from the pK_a values of 14.3 for 2'-chloroethanol and 12.9 for 2',2'-dichloroethanol, relative to the pK_a of 16 for ethanol (Jencks & Regenstein, 1976; e.g., pK_a^{rel}(-F) = 14.3 - 16 = -1.7). We are aware of no analogous values for fluoro derivatives. The values for the fluoro derivatives are expected to be similar to those for the chloro derivatives because of the similar inductive constant (σ_I) for fluoromethyl and chloromethyl substituents (Hine, 1975; Exner, 1978) and the similar pK_a values for trifluoroethanol (12.4) and trichloroethanol (12.2; Jencks & Regenstein, 1976). A limitation of this approximation is that the geometrical arrangement within the ribose ring is constrained relative to ethanol so that the effects on pK_a may differ somewhat. The value of pK_a^{rel} for 2'-OH is from the pK_a of the ribose of several nucleosides of ~12.5 (Izatt et al., 1965; Ts'o, 1974; Johnson et al., 1988) relative to that of 16 for ethanol, giving $pK_a^{rel} = 12.5 - 16 = -3.5$. This value is expected to be too negative relative to that of the fluoro derivatives, because the adenine substituent and the other oxygen atoms of ribose presumably also contribute to lowering this observed pK_a relative to ethanol and because it is thought that the 2'-hydroxy group is deprotonated before the 3'-hydroxyl group. This uncertainty is depicted by the arrow in the figure. The pK_a of 14.8 for ethylene glycol (Jencks & Regenstein, 1976) is expected to give an upper limit for pK_a^{rel} for the -OH ($pK_a^{rel} \leq 14.8 - 16 = -1.2$); the actual value may be more negative because the 2'-hydroxyl groups of ethylene glycol are not oriented for formation of an intramolecular hydrogen bond as in the ribose ring (Izatt et al., 1965; Rohrer & Sundaralingam, 1970). Thus, the slope may be slightly more negative than that depicted, or the point for 2'-OH may exhibit a positive deviation relative to the other substituents (see Discussion).

in concert (Figure 4). Such "linear free energy relationships" correlate reactivity [$\log(k_{cat}/K_m)^G_{rel}$] with a property of the reactants, in this case, the pK_a of the product ($pK_{leaving\ group}$; Jencks, 1969; Lowry & Richardson, 1981). The pK_a can be viewed as a measure of the stability of the 3'-oxyanionic form of the product, so that the correlation shows that reactivity increases markedly as the substituents stabilize the oxyanionic form of the leaving group, with a slope of $\beta_{leaving\ group} \approx -0.8$ (see Discussion). The precise value of the slope of this plot is dependent on the knowledge of the pK_a values of the 3'-hydroxyl group of the various sugars, for which there is considerable uncertainty (see Figure 4 legend and Discussion).

DISCUSSION

Perturbation of a single functional group of the oligonucleotide substrate, the 2'-substituent of U(-1) (Figure 1), changes the rate of the *Tetrahymena* ribozyme reaction by large amounts. These data provide an understanding of why it is important to have ribose at the cleavage site. Except for the bulky 2'-*O*-methyl group, the 2'-substituents of U(-1) affect cleavage of the bound oligonucleotide substrate, rather than binding of the oligonucleotide, and the rate effects

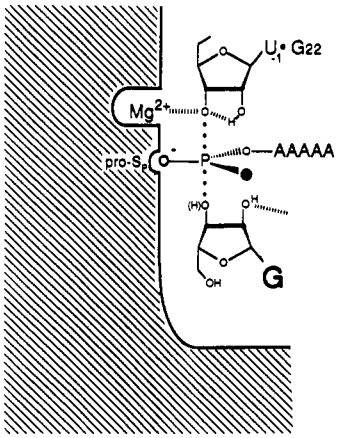

FIGURE 5: Transition-state model for phosphoryl transfer catalyzed by the *Tetrahymena* ribozyme. The reaction is depicted as an in-line displacement, because of evidence that the reaction proceeds with inversion of configuration about the phosphoryl group (McSwiggen & Cech, 1989; Rajagopal et al., 1989). Evidence for the interactions with the phosphoryl group undergoing transfer shown here come from the following sources. Data presented herein suggest the absence of protonation of the 3′-oxygen atom in the transition state as well as the presence of the intramolecular hydrogen bond between the 2′-hydroxyl group and the 3′-oxygen atom. Piccirilli et al. (1993) have obtained evidence for a direct metal ion interaction with the 3′-oxygen atom from a change in metal ion specificity for the reaction upon substituting sulfur for the 3′-oxygen. The large rate decrease ("thio effect") upon replacement of the *pro-S*$_P$, but not *pro-R*$_P$, nonbridging phosphoryl oxygen atom by sulfur suggests that there is an interaction with the *pro-S*$_P$ oxygen atom (Herschlag et al., 1991; J. A. Piccirilli and T.R.C., unpublished results); the nature of the ribozyme or ribozyme-associated functional group responsible for this interaction has not been identified. The preliminary pH dependence of Figure 2 is consistent with deprotonation of the 3′-oxygen atom of guanosine prior to or during the reaction (see Discussion) so that this proton is put in parentheses. Removal of the 2′-hydroxyl group of guanosine in the self-splicing reaction results in an even larger rate decrease than removal of the 2′-hydroxyl of U(−1) in the ribozyme reaction (Bass & Cech, 1986; Tanner & Cech, 1987; G. Narlikar and D.H., unpublished results), suggesting an important yet-unidentified role of this group.

correlate with the pK_a of the leaving group (Figure 4). The fast reaction with ribose at the cleavage site can therefore be largely explained by its greater intrinsic reactivity; i.e., the 3′-oxyanion of ribose is a better leaving group than that of deoxyribose. It remains possible that there are also specific interactions between the ribozyme and the 2′-hydroxyl of ribose that are responsible for some of the difference in reactivity. The linear free energy relationship of Figure 4 further suggests that the 3′-oxygen of U(−1) is not protonated in the transition state. Thus, it is concluded that the ribozyme does not employ general acid catalysis (Figure 5).

The Effect of Electron-Withdrawing 2′-Substituents. The 70-fold faster reaction of the 2′-F-substituted substrate than that with 2′-H [$(k_{cat}/K_m)^G_{rel}$, Table II] is consistent with an inductive effect. That is, an electron-withdrawing group such as the fluorine atom can speed a reaction in which there is development of negative charge on a nearby atom, such as the 3′-oxygen atom (Figure 5; Jencks, 1969; Lowry & Richardson, 1981). Conversely, glycosidic bond cleavage by glycogen phosphorylase is *slowed* by substitution of fluorine for hydrogen on the sugar that undergoes nucleophilic attack, consistent with development of *positive* charge in an oxycarbonium-like transition state (Street et al., 1989).

The observed faster reaction with 2′-F is also consistent with the fluorine atom accepting a hydrogen bond from a group in the active site or interacting with a metal ion in the active site [see Herschlag et al. (1993) and references therein]. However, the hydrogen bond or metal ion interaction would need to be absent or much weaker in the ground state to account for the specific transition-state effect (Table II), so that a conformational change in going from the ground state to the transition state would be required for this model to obtain. In contrast, the observed expression of the effects of the 2′-F (and -F$_2$ and -OH; see below) substituent in the transition state and not in the E·S ground-state complex is predicted directly from an inductive effect.

The alternative hydrogen bonding/metal ion model is further argued against by the additional 5-fold increase in the rate of cleavage upon introduction of a second 2′-fluorine [-F$_2$; $(k_{cat}/K_m)^G_{rel}$, Table II]. Addition of a second 2′-fluorine atom would be expected to increase an inductive effect, but decrease the strength of a hydrogen bond or metal ion coordination to the first 2′-fluorine. Thus, the additional 5-fold increase in the rate of cleavage upon introduction of the second 2′-fluorine [-F$_2$; $(k_{cat}/K_m)^G_{rel}$, Table II] supports the occurrence of an inductive effect and argues against the alternative hydrogen bonding model. The second fluorine atom is in a position normally occupied by a hydrogen atom so that no favorable interaction with the ribozyme would be expected. Further, addition of the second fluorine enhances the rate of the cleavage step despite causing weaker binding of the oligonucleotide substrate (K_d^S, Table II). There is also an unfavorable effect from -F$_2$ substitution at U(−3) (see Results). Thus, the rate enhancement from addition of the second fluorine atom may be smaller than that from addition of the first because of unfavorable interactions or unfavorable alignment introduced with this substituent.

The Linear Free Energy Correlation Suggests the Absence of General Acid Catalysis. The data described above suggest that stabilizing the oxyanionic form of the leaving group causes a large increase in the rate of cleavage by the ribozyme. If there were protonation of the 3′-oxygen atom of U(−1) in the transition state, then most or all of a potential rate enhancement from stabilization of the oxyanionic form of the leaving group would be not be expected, because the proton would neutralize the charge buildup on the 3′-oxygen atom. The rate enhancements from the 2′-F, -F$_2$, and -OH substituents relative to -H therefore suggest that the ribozyme does not utilize general acid catalysis.

The effect on leaving group stability is crudely quantitated in the linear free energy relationship of Figure 4. The slope of $\beta_{leaving\ group} = (\delta \log k/\delta\ pK_{leaving\ group}) \approx -0.8$ is similar to that of $\beta_{leaving\ group} \approx -1$ for nonenzymatic reactions of phosphate diesters (Kirby & Younas, 1970a,b). This is consistent with an absence of general acid catalysis, as protonation of the leaving group would be expected to lessen this slope. For example, there is only a small dependence of the rate of hydrolysis on the pK_a of the leaving group for reactions of phosphate monoester monoanions ($\beta_{leaving\ group} \approx -0.3$), which are thought to occur with protonation of the leaving group oxygen atom; in contrast, the reactions of the dianions, which lack this proton, are strongly dependent on the pK_a of the leaving group ($\beta_{leaving\ group} \approx -1.2$; DiSabato & Jencks, 1961; Jencks, 1962; Kirby & Varvoglis, 1967).

The conclusion that the ribozyme does not provide general acid catalysis is supported by the findings of Piccirilli et al. (1993). They observed a switch in metal ion specificity from Mg^{2+} to Mn^{2+} upon conversion of the 3′-oxygen atom of U(−1) to sulfur. This specificity switch strongly suggests that the 3′ atom is directly coordinated by the metal ion in the transition state (Figure 5). General acid catalysis in conjunction with metal ion coordination would be surprising since

protonation would significantly weaken the metal ion/3′-oxygen interaction. For example, Mg^{2+} binds ~10^3-fold more strongly to HO^- than to H_2O, as indicated by the pK_a of $Mg^{2+}(H_2O)$ of 12.4 compared to the pK_a of water of 15.7 (Baes & Mesmer, 1976). Finally, the large slope in the correlation of Figure 4 is consistent with metal ion coordination to the 3′-oxygen atom, as coordination by Mg^{2+}, unlike a covalent bond to a proton, has little or no effect on the slope of linear free energy relationships for related reactions [e.g., Herschlag and Jencks (1989)].

It is interesting to note that use of $(k_{cat}/K_m)^S$, the second-order rate constant for the reaction E·G + S → [E·G·S]‡, rather than $(k_{cat}/K_m)^G$, the second-order rate constant for the reaction E·S + G → [E·G·S]‡, in a linear free energy relationship gives $\beta_{leaving\ group} \approx 0$ (Table II; plot not shown). This is because the rate-limiting step for $(k_{cat}/K_m)^S$ is binding of the oligonucleotide substrate, not the chemical cleavage event (Table II; Herschlag & Cech, 1990a). In the absence of this knowledge, the value of $\beta_{leaving\ group} \approx 0$ might have been considered evidence *for* general acid catalysis.

Substituent effects have been used to obtain linear free energy relationships for several classes of reactions catalyzed by protein enzymes. For example, the dependence on leaving group pK_a of the cleavage of a series of uridine 3′-phosphate aryl esters by ribonuclease A to give the 2′,3′-cyclic phosphate product was compared to the dependence for the nonenzymatic reaction [Davies et al., 1988; for additional examples, see Nath and Rydon (1954) and Martin et al. (1985)].

An Intramolecular Hydrogen Bond with the 2′-Hydroxyl Group. The 2′-OH substituent gives ~10-fold faster cleavage than -F (Table II), despite the smaller inductive effect of -OH than -F (Hine, 1975; Lowry & Richardson, 1981; Exner, 1978). This deviation suggests that there is an additional stabilizing mechanism with the 2′-hydroxyl group present. In contrast to this lack of correlation with the inductive effect, the rate constants for cleavage of the 2′-OH and 2′-F substrates correlate reasonably well with the estimated pK_a values in Figure 4 (see above). The simplest explanation for the enhanced reactivity of the 2′-OH substrate is that this group donates a hydrogen bond intramolecularly to the 3′-$O^{\delta-}$ in the transition state (Figure 5), an interaction that would also lower the pK_a, maintaining the correlation of Figure 4. Despite the expectation that the geometry of such an intramolecular hydrogen bond would be suboptimal, the following argue in favor of such hydrogen bonding. The 3′-$O^{\delta-}$ may be the strongest hydrogen bond acceptor in the active site because it is more basic than potential hydrogen bond acceptors on the ribozyme, which, in the absence of perturbing influences, have pK_a values of <~4. In addition, rotation of the hydroxyl group about the C2′–O2′ bond to allow hydrogen bond donation to another moiety in the active site would create an unfavorable charge–dipole interaction between the 3′-$O^{\delta-}$ and the 2′-OH, thereby inhibiting formation of the transition state. Finally, the X-ray crystal structure of 2′-amino-2′-deoxyadenosine provides precedent for an intramolecular hydrogen bond with this geometry (Rohrer & Sundaralingam, 1970), though the presence of a bridging water molecule in the ribozyme reaction remains possible.

The partial negative charge on the 3′-oxygen in the transition state, but not in the ground state, can account for the stabilization from the 2′-OH specifically in the transition state. The contribution from the 2′-OH is only ~3-fold in the ground state (relative to -H; Table II). This hydrogen bond would also be expected to be present for the reaction in solution, thereby providing no expectation of a rate advantage for the ribozyme-catalyzed reaction from this 2′-hydroxyl group.

Formation of an intramolecular hydrogen bond to the 2′-hydroxyl group in the transition state requires that the metal ion interact with the 3′-oxygen atom from outside of the minor groove, the position of the only remaining lone pair of the 3′-oxygen atom if a roughly helical geometry is maintained in the transition state (Figure 5). This provides an additional constraint for modeling the ribozyme's active site [e.g., Michel and Westhof (1990)].

A somewhat different model for the interactions of the 2′-hydroxyl group is suggested if the relative pK_a of the 3′-hydroxyl is closer to its upper limit of ~−1.3 in Figure 4. With this value, the point for 2′-OH exhibits a positive deviation relative to the other substituents. Such a deviation could arise from a specific interaction, such as a ribozyme functional group (or bound water) that donates a hydrogen bond to the 2′-hydroxyl moiety. This functional group could position and strengthen the transition-state hydrogen bond between the 2′-hydroxyl group and the 3′-$O^{\delta-}$. However, the absence of ground-state stabilization with the 2′-F substituent (Table II), which might act as a hydrogen bond acceptor [Withers et al., 1988; see also Herschlag et al. (1993)], provides no indication of such an interaction. A deviation could also arise in the absence of a specific interaction from, for example, limited access of water to 2′-substituents in the active site, resulting in different effects of solvent in the active site than in solution. In summary, the data suggest that the 2′-hydroxyl group donates a hydrogen bond to the 3′-$O^{\delta-}$ in the transition state, though additional or alternative interactions remain possible.

A Steric Effect from the 2′-Methoxy Group. The -OCH_3 group gives an inductive effect similar to that of -OH and greater than that of -H (Hine, 1975). However, this substituent renders cleavage by the ribozyme slower than either 2′-OH or -H (see Results). In addition, ~50-fold weaker binding is observed, whereas the other substituents have little effect on binding (Table II), and miscleavage also occurs (see Results and following section). These observations suggest that there is some steric impediment to binding from the bulky methoxy group. For example, the methoxy group could sterically interfere with a ribozyme functional group (or bound water) that donates a hydrogen bond to the 2′-hydroxyl moiety (see above). Alternatively, the methoxy group could interfere with the 2-amino group of the G residue paired with U(−1) (Figure 1); a wobble pair places the 2′-position in close proximity to this amino group, and a water molecule has been observed by X-ray crystallography to bridge the 2′-hydroxyl of U and the 2-amino of G (Holbrook et al., 1991).[4] It is also possible that the methoxy substituent disrupts other parts of the active site that do not interact with the normal 2′-OH substituent.

Accuracy of 5′ Splice Site Selection. The abberrant cleavage of −1m,rS can be used to estimate the fidelity of the ribozyme for choosing the correct phosphodiester bond for cleavage, that corresponding to the 5′ splice site in the self-splicing reaction (Figure 1). The substrate −1m,rS forms abberrant products with a rate constant of ~4 min^{-1} (from

[4] It should be noted that an additional interaction of the 2′-hydroxyl group in the active site is not inconsistent with effects of this 2′-substituent in the active site that mirror the effects in solution. An absence of a water or other hydrogen bond donor to the oxygen atom of the 2′-OH in the active site might be destabilizing relative to the solution reaction, if a water molecule in solution participates in such an interaction. As stated above, it also remains possible that there are additional interactions, such as an orienting and polarizing hydrogen bond donated to the oxygen atom of the 2′-OH, which contributes to catalysis.

the E·G·S complex at pH 7 and 50 °C; data not shown). We assume that the 2′-substituent at U(−1) does not affect the stability of these alternative binding modes relative to free ribozyme and substrate and that −1m,rS and rS are cleaved at the same rate when docked in an alternative register. According to this model, it is the 50-fold weaker binding of −1m,rS than of rS in the normal register (i.e., $K_d^{-1m,rS} = \sim 50 K_d^{rS}$; Figure 3 and Table II) that allows −1m,rS to populate the alternative registers. Abberrant cleavage of rS is then predicted to be ~50-fold slower than that of −1m,rS because rS will populate the abberrant binding mode 50-fold less. This gives a value of $k \approx 4\ min^{-1}/50 = 0.08\ min^{-1}$. This rate constant is ~5000-fold smaller than the calculated rate constant of $k_c \approx 350\ min^{-1}$ for cleavage of rS at the correct site (Herschlag & Cech, 1990a), suggesting a preference of ~5000-fold for the correct site relative to the abberrant sites. This value repesents fidelity in the reaction catalyzed by the L-21 *Sca*I ribozyme; the value may be different in the self-splicing reaction.

The pH Dependence. The pH dependence of Figure 2 was initially determined to establish appropriate conditions for comparisons between different substrates (see Results and Figure 2 legend). In addition, the slope of 1 suggests that a proton is lost in the transition state. This is consistent with a reaction mechanism in which the 3′-oxygen of G is deprotonated prior to nucleophilic attack, perhaps with stabilization by coordination to Mg^{2+}. The pH dependence is also consistent with a mechanism in which a ribozyme or ribozyme-bound functional group of $pK_a \geq 8$ acts as a general base (Knowles 1976), or even a mechanism involving a pH-dependent conformational change (Kao & Crothers, 1980). (This last possibility appears to be unlikely in this case, as described in the next section.) We expect that ribozyme reactions that are first order in [HO^-] will typically be limited by the chemical step.

The Rate-Limiting Step for Reaction of the E·S·G Ternary Complex. The chemical step as defined herein includes the actual chemical cleavage as well as any associated conformational changes of the E·S·G ternary complex. Previously, we have shown that the thio effect (i.e., the rate effect from substitution of a sulfur atom for a nonbridging phosphoryl oxygen atom at the cleavage site) for the reaction of rS is consistent with rate-limiting or partially rate-limiting chemical cleavage. However, a rate-limiting conformational step that coincidentally exhibited a similar thio effect could not be eliminated (Herschlag et al., 1991). The linear free energy relationship of Figure 4 strongly suggests (but does not prove) that the chemical cleavage is indeed rate-limiting. A series of coincidences would be required to otherwise account for these data. In addition, the pH dependence of Figure 2 gives the behavior expected for a simple reaction scheme with the actual chemical cleavage rate-limiting.

Conversely, the similar rate of mRNA splicing with 2′-H, -OH, and -OCH_3 at the 5′ splice site (Moore & Sharp, 1992) might indicate that the chemical cleavage step is not rate-limiting. Otherwise, the active site would require both a general acid catalyst, rendering 2′-H and -OH similar in reactivity, and an absence of functional groups that are positioned nearby the 2′ group, rendering the reaction insensitive to inhibition from the bulky 2′-*O*-methyl substituent.

The 2′-Hydroxyl Effect Suggests a General Strategy for Specific Transition-State Stabilization in Enzymatic Reactions. The hydrogen bond depicted in Figure 5 and described above is strengthened in the transition state due to the accumulation of charge on the oxygen atom as the bond to the phosphorus atom is broken. Differential stabilization between the ground state and the transition state is crucial for catalysis. This can be understood as follows: an "enzyme" that stabilized the ground state and transition state equally would leave the same barrier for the reaction E·S → [E·S]* as for the reaction S → S* and would therefore *not* be an enzyme (Jencks, 1980).

Enzymes may in a variety of instances take advantage of changes in hydrogen bond strength that occur as the reaction procedes, thereby increasing the rate of reaction of the Michaelis complex (i.e., a "k_{cat} advantage"[5]). This catalytic strategy can be referred to as "differential hydrogen bonding". In all reactions there is a change in electron distribution as the reaction proceeds. In many cases this leads to accumulation of charge [or loss of charge (see below)] on potential hydrogen bond donating and accepting groups of the reactants. For example, the oxyanion hole of proteases has long been proposed to take advantage of the accumulation of negative charge on the carbonyl oxygen atom in the tetrahedral-like transition state in amide (and ester) cleavage (Robertus et al., 1972; Jencks, 1975).

As predicted, mutations in the oxyanions hole of subtilisin result in a decrease in k_{cat}, so that a protease without an oxyanion hole is not a good enzyme relative to an analogous enzyme with an oxyanion hole (Wells et al., 1986). However, there is a second distinct question to address: Does differential hydrogen bonding provide an advantage for k_{cat} *relative to the uncatalyzed reaction in aqueous solution*? In aqueous solution, a hydrogen bond to water will become stronger as the reaction proceeds, like the hydrogen bond to an active site residue of the enzyme. From this (oversimplified) viewpoint, no rate advantage relative to the reaction in aqueous solution would be expected.

Before we consider ways in which an enzyme might indeed realize this advantage in k_{cat} from differential hydrogen bonding, we first emphasize that differential hydrogen bonding is meant to describe situations in which the intrinsic strength of a hydrogen bonding group changes during the course of a reaction. The entropic advantage for the formation of multiple hydrogen bonds in an active site, relative to multiple hydrogen bonds with multiple water molecules in aqueous solution, provides an important mechanism for rate enhancement (Jencks, 1975) which can be considered separately. Potential rate enhancements from changes in hydrogen bond strength due to geometrical changes that occur during a reaction, which may also be important for specific transition-state stabilization, can also be considered separately.

We now consider two ways in which an enzyme can "use" differential hydrogen bonding to realize a k_{cat} advantage relative to the uncatalyzed reaction from differential hydrogen bonding. Related discussions are presented by Jencks (1975) and Warshel (1978, 1981) and related data by Tonge and Carey (1992).

(1) Ground-State Destabilization. This explanation falls within the realm of the "Circe effect", which describes the use of binding energy to provide an energetic advantage in the transition state ([E·S]*) that is not expressed or realized in the E·S ground state; k_{cat} is thereby increased (Jencks, 1975).

If the ground-state E·S complex is destabilized relative to free S in solution by the juxtaposition of hydrogen bonding

[5] By "k_{cat} advantage" we refer to effects on the rate constant for the reaction E·S → [E·S]*, with the transition state being that for the chemical transformation. Though this nomenclature is used for simplicity, it should be recognized that observed values of k_{cat} often represent steps other than the chemical transformation.

groups in the enzymatic active site, then a k_{cat} advantage can accrue from differential hydrogen bonding. Take as an example the active site of the ras protein. X-ray crystal structures suggest that the amide hydrogen of Gly 13 is in proximity of the β–γ bridge oxygen atom of bound GTP, in position to donate a hydrogen bond to this oxygen atom (Schlichting et al., 1990). However, linear free energy relationships with model phosphate diesters suggest that a bridging oxygen atom behaves as if there were a net *positive* charge localized on it [Bourne & Williams, 1984; this is consistent with the high stability of P–O bonds, which may involve electron donation from oxygen to phosphorus; e.g., Hobbs et al. (1953), Craig et al. (1954), Cruickshank (1961, 1964), Kirby and Warren (1967), Jones and Kirby (1984), Corbridge (1985), and Schmidt and Gordon (1985)]. Thus, in solution there may be no hydrogen bond donated from water to the β–γ bridge oxygen atom. In the active site the amide group of Gly 13 could be constrained to be juxtaposed to this oxygen atom, causing ground-state destabilization for the bound substrate relative to the unbound substrate. This destabilization would be relieved in the transition state as the β–γ bridge oxygen atom develops negative charge and a strong hydrogen bond to Gly 13 forms during GTP hydrolysis. The net result is a smaller barrier for the chemical step since the E·S ground-state complex is destabilized and the transition-state energy ([E·S]‡) is unaffected, to a first approximation [see (2) below]. This catalytic strategy requires interactions away from the site of bond formation or cleavage to force the destabilizing interaction in the ground state [Jencks (1975) and references therein; see also Tonge and Carey (1992)].

It is possible that the effect of the 2′-hydroxyl group observed in this work is accentuated beyond that expected solely from an effect on intrinsic reactivity by an active site specifically designed to destabilize the E·S complex. Such an active site could contain hydrogen bond donors to the oxygen atom of the 2′-OH that orient the hydrogen atom of the 2′-OH toward the 3′-oxygen atom bound to phosphorus; this might destabilize the ground state relative to that in a more open active site which allowed the hydrogen atom to rotate away from the (putative) partial positive charge on the 3′-oxygen atom (see references above).

(*2*) *Differential Increase in Hydrogen Bond Strength.* Consider a reaction in solution in which there is no significant hydrogen bond to atom X_S of the substrate in the ground state, but a strong hydrogen bond is donated to this atom in the transition state ($X_{S^{\ddagger}}$). An enzyme catalyzing this reaction would achieve a k_{cat} advantage by providing a hydrogen bond donor to X that is stronger than a hydrogen bond to water.[6] Furthermore, a k_{cat} advantage from differential hydrogen bonding can obtain even if there is a hydrogen bond to X from water in the ground state for free S, as long as X becomes a stronger hydrogen bond acceptor in the transition state ($X_{S^{\ddagger}}$) and the enzyme active site provides a stronger hydrogen bond donor than water. This advantage can arise because, as determined from model studies of hydrogen bonding, there is a *larger increase* in hydrogen bond strength as X is converted to $X_{S^{\ddagger}}$ when the interaction is with a stronger hydrogen bond donor (Hine, 1972; Stahl & Jencks, 1986). That is, even though the hydrogen bond from water gets stronger in the transition state, the hydrogen bond from the stronger hydrogen bond donor increases in strength even more. (Analogous effects can be obtained with a hydrogen bond donor on the substrate and a hydrogen bond acceptor in the active site.)

The above two cases suggest that enzymes can indeed take advantage of hydrogen bonding interactions that become stronger in the transition state due to charge redistribution. It is therefore suggested that differential hydrogen bonding can provide a rate advantage relative to the reaction in solution. Conversely, enzymes appear to be able to take advantage of hydrogen bonding interactions that become weaker in the transition state by omitting potential hydrogen bonding partners from the active site. The proposed catalysis of decarboxylation by destabilization of the ground-state carboxylate anion in a hydrophobic pocket provides an example (Crosby et al., 1970; Jencks, 1975).

[6] Hydrogen bond strength within a series of related molecules follows the pK_a of the functional group, with compounds having lower pK_a values acting as stronger hydrogen bond donors and compounds whose conjugate acids have higher pK_a values acting as stronger hydrogen bond acceptors (Stahl & Jencks, 1986; Hine et al., 1988).

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