Quantitating Tertiary Binding Energies of 2′ OH Groups on the P1 Duplex of the *Tetrahymena* Ribozyme: Intrinsic Binding Energy in an RNA Enzyme†

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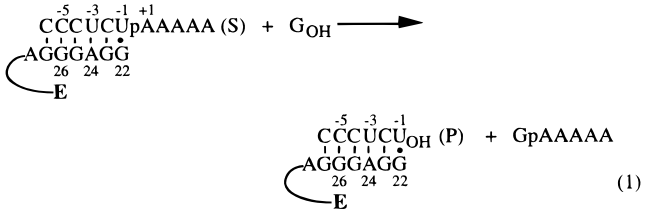
ABSTRACT: Binding of the *Tetrahymena* ribozyme's oligonucleotide substrate (S) involves P1 duplex formation with the ribozyme's internal guide sequence (IGS) to give an open complex, followed by docking of the P1 duplex into the catalytic core via tertiary interactions to give a closed complex. The overall binding energies provided by 2′ OH groups on S and IGS have been measured previously. To obtain the energetic contribution of each of these 2′ OH groups in the docking step, we have separately measured their contribution to the stability of a model P1 duplex using "substrate inhibition". This new approach allows measurement of duplex stabilities under conditions identical to those used for ribozyme binding measurements. The tertiary binding energies from the individual 2′ OH groups include a small destabilizing contribution of 0.7 kcal/mol and stabilizing contributions of up to −2.9 kcal/mol. The energetic contributions of specific 2′ OH groups are discussed in the context of considerable previous work that has characterized the tertiary interactions of the P1 duplex. A "threshold" model for the open and closed complexes is presented that provides a framework to interpret the energetic effects of functional group substitutions on the P1 duplex. The sum of the tertiary stabilization provided by the conserved G•U wobble at the cleavage site and the individual 2′ OH groups on the P1 duplex is significantly greater than the observed tertiary stabilization of S (11.0 vs 2.2 kcal/mol). It is suggested that there is an energetic cost for docking the P1 duplex into the active site that is paid for by the "intrinsic binding energy" of groups on the P1 duplex. Substrates that lack sufficient tertiary binding energy to overcome this energetic barrier exhibit reduced reactivities. Thus, the ribozyme appears to use the intrinsic binding energy of groups on the P1 duplex for catalysis. This intrinsic binding energy may be used to position reactants within the active site and to induce electrostatic destabilization of the substrate, relative to its interactions in solution.

The *Tetrahymena* ribozyme (E)[1] catalyzes a phosphoryl transfer reaction with a rate enhancement of 10^{11}-fold over the uncatalyzed reaction (eq 1; Herschlag & Cech, 1990). This rate enhancement is comparable to that observed with many protein enzymes. Jencks has proposed that protein



(1)

enzymes use the intrinsic binding energy of interactions not directly involved in the chemical transformation for catalysis (Jencks, 1975). This binding energy can be utilized for catalysis by positioning substrates within the active site, thereby reducing the entropic barrier for reaction, and by inducing substrate destabilization relative to solution. As a result, the observed binding energy of substrates is less than their intrinsic binding energy. The use of intrinsic binding energy for catalysis has been experimentally demonstrated in a number of protein enzymes (e.g., Baumann et al., 1973;

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[1] Abbreviations: E refers to the *Tetrahymena* L-21 *Sca*I ribozyme. T1 refers to ribonuclease T1. Unless specified otherwise, S refers generically to the oligonucleotide substrate, CCCUCUAAAAA, without specification of the sugar identity. P similarly refers generically to the oligonucleotide product, CCCUCU. To specify the identity of the sugar residues, the following nomenclature is used: rS and dS refer to substrates having all ribose and all deoxyribose residues, respectively. Chimeric oligonucleotides are named according to the residues that are different from the all-ribose background of rS or the all-deoxyribose background of dS. For example, −3d,rS refers to a substrate having a deoxyribose residue at position −3 (eq 1) in a background of ribose residues. Analogously, the substrate having a 2′ amino substitution at the −3 position in a background of ribose residues is referred to as −3N,rS. IGS refers to the internal guide sequence of the ribozyme (Figure 1) having the sequence $^{5'}$GGAGGG. The IGS analogs used in the substrate inhibition studies are referred to as IGS′; the different sequences of the IGS analogs are specified along with the individual experiments. *pS and *pIGS′ refer to 5′-[^{32}P]-labeled S and 5′-[^{32}P]-labeled IGS′, respectively. To refer to a specific residue within S, the residue is followed by the number of its position in parentheses (eq 1). For example, the U three residues 5′ of the cleavage site is referred to as U(−3). To refer to a specific residue in the IGS or the IGS analogs, the residue is followed by the number of its position in the full-length *Tetrahymena* intron (eq 1); the L-21 *Sca*I ribozyme begins with residue G22. MES, 2-morpholinoethanesulfonic acid; EPPS, *N*-2-(hydroxyethyl)piperazine-*N*-3-propanesulfonic acid; EDTA, ethylenedinitrilotetraacetic acid; tris, tris(hydroxymethyl)aminomethane.

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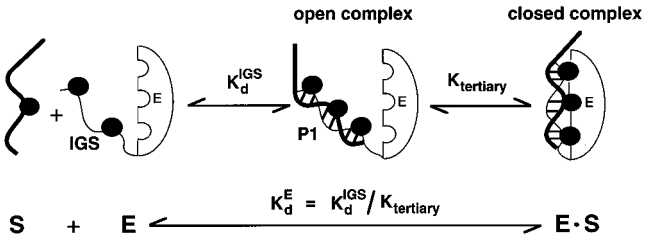


FIGURE 1: Two-step model for binding of oligonucleotide substrate (S). S (thick line) first forms the open complex by base pairing with the internal guide sequence (IGS = 5′GGAGGG) of the ribozyme (E) to give the P1 duplex. The P1 duplex then docks into the catalytic core via tertiary interactions to form the closed complex (Bevilacqua & Turner, 1991; Pyle & Cech, 1991; Bevilacqua et al., 1992; Herschlag, 1992; Herschlag et al., 1993; Strobel & Cech, 1993, 1995; Pyle et al., 1994; Knitt et al., 1994). Three of the 2′ OH groups involved in tertiary stabilization [e.g., G22, G25, and U(−3)] are shown schematically as filled circles. K_d^E is the equilibrium constant for dissociation of S from E·S. K_d^{IGS} is the equilibrium constant for dissociation of S from the open complex. $K_{tertiary}$ is the equilibrium constant for docking into the tertiary interactions. The relation between K_d^E, K_d^{IGS}, and $K_{tertiary}$ depicted in the figure holds when docking is highly favored ($K_{tertiary} \gg 1$, see eq 4, Materials and Methods).

Holler et al., 1973; Jencks, 1975; Fersht, 1987). Binding and catalytic cleavage of the *Tetrahymena* ribozyme's oligonucleotide substrate provide an opportunity to study the relationship between binding energy and catalysis in an RNA enzyme.

Initial work indicated that the ribozyme's oligonucleotide substrate (S) is bound by both base-pairing interactions with the ribozyme's internal guide sequence (IGS) as well as by tertiary interactions with the catalytic core (Sugimoto et al., 1989; Herschlag & Cech, 1990; Pyle et al., 1990). Subsequent work indicated that base-pairing and tertiary interactions were made in two separate steps (Figure 1; Bevilacqua et al., 1992, 1994; Herschlag, 1992; Li et al., 1995; Narlikar & Herschlag, 1996). The tertiary interactions were shown to involve the G·U wobble pair at the cleavage site and specific 2′ OH groups on S and IGS (Bevilacqua & Turner, 1991; Pyle & Cech, 1991; Herschlag et al., 1993; Strobel & Cech, 1993, 1995; Knitt et al., 1994; Pyle et al., 1994).

According to the two-step model for substrate binding (Figure 1), the binding energy provided by a specific group consists of a contribution to formation of the P1 duplex, and a contribution to tertiary stabilization of the P1 duplex. Here, we have quantitated the tertiary stabilization provided by individual 2′ OH groups of S and IGS. The energetic analysis herein suggests that, analogous to protein enzymes, the *Tetrahymena* ribozyme uses the intrinsic binding energy of tertiary interactions for catalysis.

MATERIALS AND METHODS

Materials. Ribozyme (E) was prepared by *in vitro* transcription and purified as described previously (Zaug et al., 1988). Ribonuclease T1 was from United States Biochemical Corp. Oligonucleotides were made by solid-phase synthesis. Several were supplied by CloneTec (Palo Alto, CA) or by the Protein and Nucleic Acid Facility at Stanford or were used and characterized in previous studies. The oligonucleotide −3N,rS was a gift from F. Eckstein. Oligonucleotides were 5′-end-labeled with approximately equimolar amounts of [γ-^{32}P]ATP using T4 polynucleotide kinase and were purified by nondenaturing polyacrylamide gel electrophoresis, as described previously (Zaug et al., 1988; Herschlag et al., 1993). The IGS analogs and CCCUCUA, which were used as substrate inhibitors in the ribozyme and ribonuclease T1 assays, respectively, were HPLC purified prior to use.

General Kinetic Methods. All reactions were single-turnover, with E in excess of 5′-end-labeled S (*pS, ∼0.1 nM), and were carried out at 50 °C in 50 mM buffer and 10 mM $MgCl_2$ (Herschlag & Cech, 1990; Herschlag et al., 1993); the pH is specified along with the individual experiments. The buffers used and their pH values at 50 °C are NaMES, pH 6.6 and 5.0, and NaEPPS, pH 8.0. The values at 50 °C are obtained from the respective values at 25 °C using correction factors obtained previously (Knitt & Herschlag, 1996). Reactions were initiated by addition of *pS following a 15 min/50 °C preincubation of E in $MgCl_2$ and buffer. The T1 reactions were also single-turnover with T1 in excess of *pIGS′ and were carried out at 50 °C in 50 mM NaMES, pH 6.6, and 10 mM $MgCl_2$. These reactions were initiated by addition of T1. For both the ribozyme and T1 reactions, 6 aliquots of 1−2 μL were removed from 20 μL reactions at specified times, and further reaction was quenched by the addition of ∼2 volumes of 20 mM EDTA in 90% formamide with 0.005% xylene cyanole, 0.01% bromophenol blue, and 1 mM Tris, pH 7.5. Substrate and product(s) were separated by electrophoresis on 20% polyacrylamide/7 M urea gels, and their ratio at each time point was quantitated with a Molecular Dynamics Phosphorimager.

Reactions were typically followed for $\sim 3t_{1/2}$ except for some very slow ribozyme reactions. End points of ∼95% and 99% were typically obtained with the ribozyme and T1 reactions, respectively, and were used in nonlinear first-order fits to the data (KaleidaGraph, Synergy Software, Reading, PA). For the slow ribozyme reactions, in which only initial rates could be measured, an end point of 95% was assumed.

Binding to the Ribozyme. The equilibrium constant for dissociation of S from E·S, K_d^E, was obtained at pH 6.6 with subsaturating guanosine (G) concentrations (0.2−50 μM; $K_d^G \approx 200$ μM; McConnell et al., 1993), with the exception of the substrate −3N,rS, for which the dissociation constant was determined at pH 5.0 and 8.0. Values of K_d^E for rS, −2d,rS, −3d,rS, and (−4)−(−6)d,rS were obtained from $K_d^E = k_{off}/k_{on}$, where k_{off} is the rate constant for dissociation of S from E·S and k_{on} is the rate constant for binding of S to E. Values of k_{off} for rS, −2d,rS, and −3d,rS were determined previously by pulse-chase experiments (Herschlag et al., 1993). The value of k_{off} for (−4)−(−6)d,rS was measured by analogous pulse-chase methods. The value of k_{on} at subsaturating G is equal to $(k_{cat}/K_m)^{S,app}$, the apparent second-order rate constant for the reaction, E + G + S → products, when all the S that binds E gets cleaved rather than dissociates from the E·S complex [see Herschlag and Cech (1990) for a more detailed description]. For rS, this was confirmed by pulse-chase experiments for the G concentrations used. For −2d,rS, −3d,rS, and (−4)−(−6)d,rS, k_{off} is comparable to the rate of reaction at subsaturating G and $(k_{cat}/K_m)^{S,app}$ does not represent k_{on} at subsaturating G. Hence for these substrates, the $(k_{cat}/K_m)^{S,app}$ value of rS was used. This is a valid extrapolation because, at saturating G, the $(k_{cat}/K_m)^S$ values for all these substrates represent k_{on} and are identical. For −1d,rS and −3N,rS, K_d^E values were obtained by following reactions with varying [E]. These experiments were carried out under conditions

such that each concentration dependence obtained reflects the true equilibrium dissociation constant, K_d^E, as described previously (McConnell et al., 1993; Knitt et al., 1994).

Substrate Inhibition To Measure Duplex Stabilities. (A) Substrate Inhibition with the Tetrahymena Ribozyme. Reactions were carried out at 50 °C, pH 6.6, with 2 mM G, 0.5−10 nM E, ∼0.1−1 nM of the appropriate *pS, and varying concentrations of the IGS analog, IGS′. [E] was always in excess of [*pS]. After preincubation of E and G in buffer and $MgCl_2$ at 50 °C, 2 μL of the appropriate 10× solution of IGS′ was added. To disrupt potential aggregates that may have accumulated during storage, aliquots of the IGS′ strand were heated to 95 °C for 5 min, in 10 mM Tris and 1 mM EDTA, and cooled to room temperature each time before use. Reactions were initiated by addition of 2 μL of *pS to give a final reaction volume of 20 μL.

Stabilities of duplexes with rP (CCCUCU) and rP variants (CCCUCC, CUCUCU and CUCUCC) were obtained by following a reaction analogous to the reverse of the reaction in eq 1 (eq 2). In these reactions, 1 mM GA and 2−50 nM

$$E + GpA + CCCUCU \rightarrow E + G + CCCUCUpA \qquad (2)$$

E were used and reactions initiated with ∼0.1 nM of the appropriate 5′-[^{32}P]-labeled rP or rP variant. Stabilities of duplexes with CCCUCC and CUCUCC were also measured by following inhibition of the miscleavage reaction. Miscleavage refers to cleavage at a site other than U(−1) and occurs when the P1 duplex docks into alternative registers within the core of the ribozyme. This results in positioning of different phosphate bonds at the cleavage site (Herschlag, 1992; Knitt et al., 1994; Strobel & Cech, 1994). For example, miscleavage of CCCUCU results predominantly in the shorter products CCCUC and CCCU due to cleavage at C(−2) and U(−3), respectively.

(B) Substrate Inhibition with Ribonuclease T1. Reactions were carried out at 50 °C, pH 6.6, 10 mM $MgCl_2$, 0.8−4.0 nM T1, ∼0.1 nM of the appropriate *pIGS′ strand, and varying concentrations of the inhibitor strand, CCCUCUA. *pIGS′ was heated to 95 °C for 5 min and cooled to room temperature before adding to the reaction mixture. Reactions were initiated by adding 2 μL of 10× T1 to give a final volume of 20 μL. Reactions were carried out in siliconized Eppendorf tubes. (Time-dependent inactivation of T1 was observed in nonsiliconized tubes, presumably due to sticking of the enzyme to the tube.)

The reaction with each IGS′ analog gives the ladder of products expected from cleavage by T1, which specifically cleaves the phosphate bond 3′ to single-stranded riboguanosine residues (Uchida & Egami, 1971). There is no detectable cleavage (<0.1%) of 5′-labeled CCCUCUA by T1 over the time scale of the substrate inhibition experiments.

(C) Inhibition Constants. For both the ribozyme and T1 assays, inhibition curves were obtained with 6 or more IGS′ or CCCUCUA (inhibitor) concentrations. The inhibitor concentrations spanned a range of 3−10-fold above to 3−10-fold below the inhibition constant. The inhibition data were normalized and fit according to eq 3 to give the

$$k_{norm} = k_{obs}/k_{obs}^{0} = 1/(1 + [\text{inhibitor}]/K_I) \qquad (3)$$

inhibition constant, K_I (see Results). In eq 3, k_{obs} is the observed rate constant and k_{obs}^0 is the rate constant in the absence of inhibitor.

(D) Control Experiments for Substrate Inhibition. For the inhibition constant (K_I) to equal the dissociation constant for the duplex between S and IGS′ ($K_d^{IGS'}$), two criteria must be met: (i) IGS′ must be in equilibrium with S and (ii) most of S must be unbound from E (see description in Results). (i) For IGS′ to be in equilibrium with S, the ribozyme must cleave S slower than the time required for S and IGS′ to reach equilibrium. The following results established that S and IGS′ reached equilibrium faster than cleavage of S by the ribozyme. (a) Good, single-phase, first-order kinetics were observed for the disappearance of the 5′-labeled oligonucleotides. (b) Varying the time of preincubation of $^{5'}$GGAGGGA with $CCCUCUA_5$ or with $CCCUCCA_5$ from zero to 60 min did not affect the amount of inhibition. (c) Accelerating the ribozyme reaction by 10-fold by increasing [E] did not affect the observed inhibition with $CCCUCUA_5$ or $CCCUCCA_5$. [The observed rate constant is linearly proportional to [E] under $(k_{cat}/K_m)^S$ conditions (see below), whereas the extent of inhibition is expected to be unaffected.] Analogous results with the T1 reactions established that free CCCUCUA and IGS′ reached equilibrium faster than cleavage of the IGS′ analogs by T1. (ii) Under $(k_{cat}/K_m)^S$ conditions, most of S is not bound to E because S is subsaturating with respect to excess E. Linear increases in the rate constants for S cleavage, with increasing E concentrations (0.5−50 nM), established that the reactions were carried out under $(k_{cat}/K_m)^S$ conditions. Analogous control experiments were done to establish $(k_{cat}/K_m)^P$ conditions for all the reverse reactions and all the miscleavage reactions. (k_{cat}/K_m) conditions for the T1 reaction were established by varying [T1] from 0.8 to 4.0 nM.

Potential complications from IGS′ aggregation were ruled out by the following results. (a) Good first-order kinetic behavior is observed for the disappearance of S at the highest IGS′ concentrations used. This suggests that IGS′ does not aggregate in a time-dependent manner. (b) The observed rate constants approach zero at high [IGS′] and fit well to a simple hyperbolic inhibition curve in all cases (eq 3). This provides evidence against aggregation of the IGS′ strand at high concentrations and also indicates that reaction of the model P1 duplex *in trans* does not contribute significantly to the observed rate constant for S cleavage. (c) The energetic consequence from mismatches and other changes was the same in different duplexes (see Results and Supporting Information).

The following results indicate that IGS′ does not interact significantly with the ribozyme. (a) Preincubating IGS′ (GGAGGGA) and E for various times (0−20 min) before addition of CCCUCUA gives the same inhibition. (b) The same dissociation constant is obtained, within error, for the P1 duplex between GGAGGGA and CCCUCUA using the ribozyme and ribonuclease T1 assays (see Results).

Calculation of $K_{tertiary}$, the Tertiary Stabilization of the Substrate. Binding of S to E involves P1 duplex formation followed by P1 docking (Figure 1). Hence, K_d^E, the equilibrium constant for dissociation of the E·S complex, is related to K_d^{IGS}, the equilibrium constant for dissociation of the P1 duplex, and $K_{tertiary}$, the equilibrium constant for P1 docking according to eq 4. The dissociation constant of the

$$K_d^E = K_d^{IGS}/(1 + K_{tertiary}) \quad (4)$$

ribozyme's P1 duplex, K_d^{IGS}, is estimated from the dissociation constant of the corresponding model P1 duplex, $K_d^{IGS'}$ (see Results). The tertiary stabilization provided by 2′ OH groups on the substrate strand is calculated for the binding of rS, whereas the tertiary stabilization provided by 2′ OH groups on the IGS is calculated for the binding of rP. The measurements here and in previous work suggest that the tertiary contributions of individual functional groups on the P1 duplex are the same for rS and rP binding (Pyle & Cech, 1991; Bevilacqua & Turner, 1991; Herschlag et al., 1993).

To obtain the values of $K_{tertiary}$ in Table 7, the values of K_d^E and $K_d^{IGS'}$ were obtained as follows. The K_d^E value of 2.5 nM for dissociation of rP from the normal ribozyme is from Narlikar et al. (1995). The K_d^E values for dissociation of rP from the modified ribozymes were calculated from the K_d^E values of Strobel and Cech (1993) measured at 42 °C, assuming that the effects of replacing 2′ OH groups with 2′ H groups are the same at 42 and 50 °C. Introducing single 2′ OH substitutions in a DNA substrate has the same effect on $(k_{cat}/K_m)^S$ at 30 and 50 °C (Herschlag et al., 1993; D. Herschlag, unpublished results; $(k_{cat}/K_m)^S$ is the second-order rate constant for the reaction, E·G + S → E + products, and consists of the steps of P1 duplex formation, P1 docking, and chemical cleavage), suggesting that the above temperature extrapolation is valid. The $K_d^{IGS'}$ values for dissociation of rP from a model P1 duplex were obtained from the respective $K_d^{IGS'}$ values for rS after accounting for a stabilizing contribution of 6-fold from stacking of the 3′ A residues of rS. The 6-fold effect is obtained from comparison of the stability of duplexes of rP (CCCUCU) and rS (CCCUCUAAAAA) with an all-ribose IGS′ (GGAGGG, Table 1).

To obtain the stability of the P1 duplex, the IGS′ analog having the sequence 5′GGAGGGA was used, which includes the 3′ A residue of the J1/2 region adjoining the ribozyme's IGS. The 3′ A residue was included because it provides additional stabilization via stacking (Freier et al., 1986) and because the ribozyme's P1 duplex has the same stability as a model P1 duplex containing this residue (Bevilacqua et al., 1992; Narlikar & Herschlag, 1996).

Oligonucleotide Concentrations. Concentrations of all the IGS′ analogs and CCCUCUA were calculated from absorbance measurements at 260 nm (room temperature, pH 7.0, 10 mM Tris and 1 mM EDTA) using extinction coefficients calculated as a sum of the extinction coefficients of the individual bases.

Estimation of Error Limits. There were variations of rate constants of up to ~2-fold between experiments. Ratios of rate constants varied less within the same experiment. Values of K_d^E and $K_d^{IGS'}$ obtained from the rate measurements varied by ±50% in independent experiments. However, the variation in the ratios of $K_d^{IGS'}$ values and K_d^E values obtained in independent experiments was typically much less (<±10%, corresponding to ±0.06 kcal/mol at 50 °C).

RESULTS

Dissecting the Energetic Contributions from the Two Steps of Substrate Binding. To obtain the tertiary binding energy provided by a functional group on the P1 duplex from its contribution to overall binding, it is necessary to know the contribution of the group toward stabilizing the open complex (Figure 1). Comparisons of transiently detected or thermodynamically stable open complexes with model duplexes have indicated that S is bound in the open complex with the stability predicted from base-pairing interactions with the IGS (Bevilacqua & Turner, 1992; Narlikar & Herschlag, 1996). Hence, the energetic contribution of a functional group to the stability of the open complex can be obtained from its contribution to the stability of a model P1 duplex.

In the first section we describe a new approach referred to as "substrate inhibition" for measuring the stabilities of model duplexes. In the subsequent sections, we use the

Table 1: Comparison of Duplex Stabilities Obtained by Substrate Inhibition, Optical Melting Studies, and Nearest Neighbor Rules[a]

Duplex	Duplex Stabilities (μM)		
	Substrate Inhibition ($K_d^{IGS'}$)	Nearest Neighbor Rules (K_d^{NN})	Temperature Melting Studies (K_d^{Tm})
5′CCCUCU AGGGAGG	3.5	10	1.5
5′CUCUCU AGGGAGG	940	520	350
5′CCCUCC AGGGAGG	0.48	2.0	0.11
5′CUCUCC AGGGAGG	69	92	-
5′CCC UCU GGGAGG	7.0	45	11
5′CCC UCU GGGAGA	6.7	53	-
5′CCC UCU AGGAGG	87	320	-
5′CCC UCU AGGAGA	200	370	-
5′CCC UCUA GGGAGG	1.4	11	-
5′CCC UCUA GGGAGA	1.5	28	-
5′CCC UCUA AGGAGG	16	78	-
5′CCC UCUA AGGAGA	22	200	-
5′CCC UCUAAAAA GGGAGG	1.1	11	-

[a] Duplex stabilities at 50 °C. $K_d^{IGS'}$ values are from substrate inhibition measurements at 10 mM $MgCl_2$ and 50 mM NaMES, pH 6.6. K_d^{NN} values are calculated for 1 M NaCl, pH 7.0, using nearest neighbor rules (Freier et al., 1986; Serra & Turner, 1995). For calculating energetic effects due to the 3′ A overhang in $\begin{matrix}UA\\G\end{matrix}$, the average of the energetic effects of 3′ A overhangs in $\begin{matrix}UA\\A\end{matrix}$ and $\begin{matrix}CA\\G\end{matrix}$, 0.9 kcal/mol, was used (Serra & Turner, 1995). K_d^{Tm} values are calculated using the ΔH and ΔS parameters obtained from optical melting studies at 10 mM $MgCl_2$, 10 mM NaCl, 50 mM Tris, pH 7.6, in Pyle et al. (1994). (−) refers to "not determined". For the substrate inhibition studies, the S or P strand has a 5′-phosphate and the IGS analog has a 5′ OH. The duplex stabilities obtained from nearest neighbor calculations and optical melting measurements are for duplexes with 5′ OH groups on both strands. In control experiments using substrate inhibition, a 5′-phosphate was shown to contribute less than 2-fold to the stability of the duplex relative to a 5′ OH.

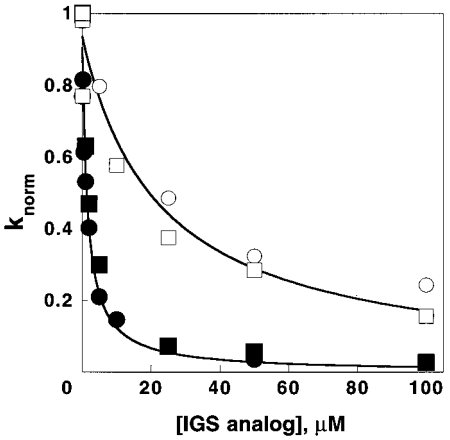


FIGURE 2: The energetic effect of replacing a C·G pair (closed symbols) with a C·A pair (open symbols). Stabilities of the duplexes $\overline{\text{C}}\overset{\text{CCUCUA}}{\underline{\text{G}}\text{GGAGG}}$ and $\overline{\text{C}}\overset{\text{CCUCUA}}{\underline{\text{A}}\text{GGAGG}}$ were measured by substrate inhibition. Single-turnover reactions of *pCCCUCUA with G were catalyzed by E (50 °C, pH 6.6, 10 mM $MgCl_2$) in the presence of varying concentrations of the IGS analog ($^{5'}$GGAGGG or GGAGGA). The rate constants are normalized such that $k_{norm} = 1$ in the absence of the IGS analog (see Materials and Methods). Different symbols represent results from independent experiments. The lines are nonlinear least-squares fits to the data and give $K_d^{IGS'} = 1.4$ and 16.0 μM, respectively, for $\overline{\text{C}}\overset{\text{CCUCUA}}{\underline{\text{G}}\text{GGAGG}}$ and $\overline{\text{C}}\overset{\text{CCUCUA}}{\underline{\text{A}}\text{GGAGG}}$.

method to define the tertiary stabilization provided by the individual 2′ OH groups of S and IGS.

Substrate Inhibition as a Method To Measure Duplex Stabilities. Conventional competitive inhibitors decrease the observed reaction rate by binding to the enzyme, thereby inhibiting binding and reaction of the substrate. Here, a different type of competitive inhibition is used in which the inhibitor instead binds to the substrate and prevents its reaction. In the present case, an oligonucleotide strand having the sequence of the ribozyme's IGS (IGS′) binds to S and inhibits the ribozyme-catalyzed reaction (eq 5). Under

$$\begin{array}{ccc} \text{E·G} + \text{*pCCCUCUA} & \xrightarrow{k_{cat}/K_m} & \text{E} + \text{CCCUCU} + \text{GA} \\ \pm\text{GGAGGGA} \Big\Downarrow & K_I = K_d^{IGS'} & \\ \text{*pCCCUCUA} & & \\ \text{AGGGAGG} & & \end{array} \quad (5)$$

conditions where S is not complexed with E and is in equilibrium with IGS′, the inhibition constant, K_I, equals the dissociation constant $K_d^{IGS'}$ for the model P1 duplex between S and IGS′ (eq 5).

A series of general control experiments, described in Materials and Methods, were carried out as initial tests of the validity of this method. The results lead to the following conclusions: (i) Under the reaction conditions employed, S is in equilibrium with IGS′ and is not complexed to a significant extent with E. (ii) There is no aggregation of the IGS′ strand at the concentrations used. (iii) There is no detectable cleavage of the model P1 duplex *in trans*. (iv) There is no detectable interaction between IGS′ and the ribozyme.

In a further series of control experiments, the stabilities of a series of different duplexes were measured and compared. Sample inhibition curves for two of the duplexes are shown in Figure 2. The results are summarized in Tables 1 and 2 and in the Supporting Information and indicate the following. (i) The duplex stabilities measured by substrate inhibition are in reasonable agreement with those obtained from optical melting studies and those calculated from nearest neighbor rules (Table 1, $K_d^{IGS'}$, $K_d^{T_m}$, and K_d^{NN}, respectively). For example, the stabilities of $\overset{\text{CCCUCU}}{\text{AGGGAGG}}$, $\overset{\text{CCCUCC}}{\text{AGGGAGG}}$, and $\overset{\text{CUCUCU}}{\text{AGGGAGG}}$ measured by substrate inhibition at 50 °C are within 4-fold of the values obtained from nearest neighbor calculations and T_m measurements. In some cases, values of $K_d^{IGS'}$ ($\overset{\text{CCCUCUA}}{\text{AGGAGA}}$) and $K_d^{T_m}$ ($\overset{\text{CCCUCC}}{\text{AGGGAGG}}$) varied by ~10−20-fold from the respective K_d^{NN} values, presumably because of differences in the buffer and salt or limitations in the accuracy of the nearest neighbor rules. (ii) There appear to be no complications due to a particular ribozyme assay or ribozyme concentration. The duplex stabilities of $\overset{\text{CCCUCC}}{\text{GGGAGG}}$ and $\overset{\text{CUCUCC}}{\text{AGGGAGG}}$ were measured by two different ribozyme assays: the reverse reaction and miscleavage reaction (see Materials and Methods). These assays used different E concentrations (with CCCUCC, [E] = 2 and 5 nM for the miscleavage reaction and reverse reaction, respectively; with CUCUCC, [E] = 20 and 50 nM for the miscleavage reaction and reverse reaction, respectively). Nevertheless, the stabilities of the duplexes measured by the two different assays are the same, within experimental error (Table 2). (iii) The effect on duplex stability of several specific changes is the same, within experimental error, in the context of different duplexes: changing a 5′ terminal $\overset{\text{CC}}{\text{GG}}$ to a $\overset{\text{CC}}{\text{AG}}$ has destabilizing effects of 10−30-fold; changing a 3′ terminal $\overset{\text{CU}}{\text{GG}}$ to a $\overset{\text{CU}}{\text{GA}}$ has effects of 1−2-fold; changing a 3′ terminal $\overset{\text{CU}}{\text{GG}}$ to a $\overset{\text{CC}}{\text{GG}}$ has stabilizing effects of 7−13-fold; and changing an internal $\overset{\text{UC}}{\text{GG}}$ to a $\overset{\text{CC}}{\text{GG}}$ has effects of 140−270-fold (Supporting Information). Similarly, the additional stacking energy obtained from a 3′ A overhang in $\overset{\text{CUA}}{\text{GG}}$ is the same, within error (5-fold), in the context of two different duplexes (Supporting Information). These results provide additional evidence against aggregation of IGS′ at high concentrations, because different ranges of [IGS′] are used in the context of duplexes with different stabilities. For example, $\overset{\text{CCCUCU}}{\text{AGGGAGG}}$ is a more stable duplex compared to $\overset{\text{CUCUCU}}{\text{AGGGAGG}}$ ($K_d^{IGS'}$ = 3.5 and 940 μM, respectively). Hence, whereas 0.05−35 μM of GGAGGGA was used to measure the energetic effect of changing a 3′ terminal $\overset{\text{CU}}{\text{GG}}$ to a $\overset{\text{CC}}{\text{GG}}$ in $\overset{\text{CCCUCU}}{\text{AGGGAGG}}$, 7−2400 μM GGAGGGA was used to measure the energetic effect of the same substitution in $\overset{\text{CUCUCU}}{\text{AGGGAGG}}$ (Supporting Information). The energetic effects of the substitutions described above are in reasonable agreement (within 2−5-fold) with the

Table 2: Duplex Stabilities Measured by Two Different Ribozyme Assays[a]

Duplex	$K_d^{IGS'}$ (μM)	
	Reverse Reaction	Miscleavage
$^{5'}\overset{\text{CCCUCC}}{\text{GGGAGG}}$	1.4	1.2
$^{5'}\overset{\text{CUCUCC}}{\text{AGGGAGG}}$	69	48

[a] $K_d^{IGS'}$ values were measured at 50 °C, 50 mM NaMES, pH 6.6, 10 mM $MgCl_2$, by substrate inhibition, using either the reverse reaction of the ribozyme (eq 2) or the miscleavage reaction, as described in Materials and Methods.

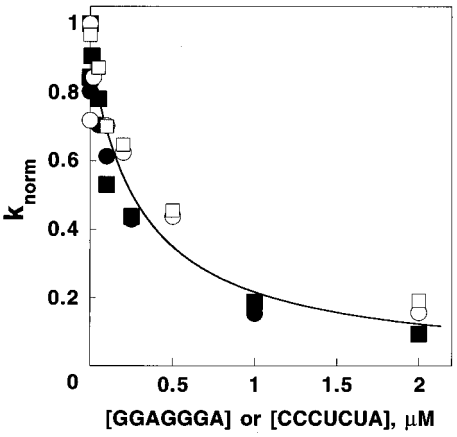
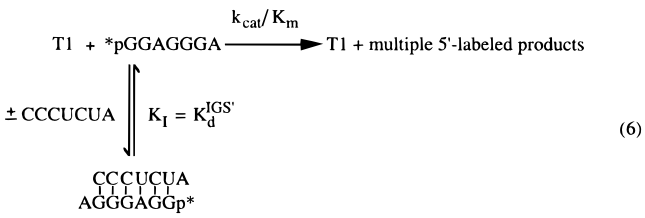


FIGURE 3: Ribonuclease T1 and the *Tetrahymena* ribozyme assays give the same duplex stabilities in substrate inhibition experiments. The stability of $^{CCCUCUA}_{AGGGAGG}$ was measured using T1 (closed symbols) and the *Tetrahymena* ribozyme (open symbols) reactions. T1 reactions were carried out with 0.1 nM *pGGAGGGA in the presence of varying concentrations of CCCUCUA and the ribozyme reactions with 0.1 nM *pCCCUCUA in the presence of varying concentrations of GGAGGGA. The rate constants are normalized such that $k_{norm} = 1$ in the absence of the inhibitor (see Materials and Methods). Different symbols represent results from independent experiments. The line represents a nonlinear least-squares fit to the combined data with T1 and the ribozyme and gives $K_d^{IGS'} = 0.30$ μM ($r = 0.96$). The separate fits (not shown) to the T1 and ribozyme results give $K_d^{IGS'} = 0.22$ and 0.43 μM, respectively.

energetic effects predicted from nearest neighbor rules and T_m measurements (Supporting Information and Table 1).

In summary, the control experiments described here and in the Materials and Methods establish the validity of substrate inhibition as a method to measure equilibrium duplex stabilities.

Substrate Inhibition Using Ribonuclease T1. In principle, substrate inhibition can be generalized for use in other enzymatic systems and even in nonenzymatic systems. To extend this method to another enzyme system, we used ribonuclease T1 (T1) to measure the duplex stabilities of a series of model P1 duplexes. T1 preferentially cleaves the phosphate bond 3′ to a riboguanosine residue in single-stranded oligonucleotides (Uchida & Egami, 1971). Hence, in contrast to the ribozyme assay, the 5′-labeled strand that gets cleaved is the IGS analog, *pGGAGGGA. The inhibitor strand is CCCUCUA (eq 6). The stability of $^{CCCUCUA}_{AGGGAGG}$ was



(6)

the same, within error, when measured by the ribozyme or T1 assay (Figure 3; $K_d^{IGS'} = 0.22$ and 0.43 μM by T1 and the ribozyme assay, respectively).[2]

In another comparison of the two assays, the stabilities of model P1 duplexes that had single 2′ H substitutions in the IGS′ strand were measured. The energetic effect of each 2′ H substitution on duplex stability is the same, within error, when measured with the ribozyme and T1 assays (Table 3). In summary, the results demonstrate that substrate inhibition can be extended to other enzyme systems. Complications that may be specific to the ribozyme system are also ruled out.

Advantages and Limitations of Substrate Inhibition. Substrate inhibition has certain advantages over conventional T_m measurements. Duplex stabilities can be measured directly under reaction conditions and at the temperature of interest. In some cases, this eliminates the need to obtain duplex stabilities by extrapolation of data obtained at other temperatures. In addition, compared to T_m measurements, lower concentrations of oligonucleotides can be used, so that stronger binding at lower temperatures can be directly measured. Finally, unlike T_m measurements, this method does not require a special apparatus.

This method can also be used to obtain the rate constant for duplex formation on an enzyme relative to that for duplex formation in solution. Upon addition of labeled substrate to a solution of inhibitor and enzyme, its initial partitioning between binding to the inhibitor and binding to the enzyme is determined by the relative rate constants for binding of substrate to the inhibitor and enzyme and the relative concentrations of inhibitor and enzyme. Experiments measuring partitioning of $CCCUCCA_5$ between binding to GGAGGGA and to E using different ratios of GGAGGGA and E concentrations gave no burst of ribozyme activity, consistent with faster binding to GGAGGGA. The results suggested that the rate constant for duplex formation in solution is at least 10-fold larger than the rate constant for duplex formation on the ribozyme (50 °C, data not shown). As discussed above, the P1 duplex has the same stability in solution and on the ribozyme. Hence, the rate constant for dissociation of a model P1 duplex in solution will also be correspondingly larger than the rate constant for dissociation of the ribozyme's P1 duplex. This confirms and extends previous results in which duplex formation between a shorter oligonucleotide, CCUCU, and the ribozyme's IGS was determined to be ~10-fold slower (15 °C) than the rate constant for duplex formation between CCUCU and an oligonucleotide having a modified IGS sequence, GGAGGA (Bevilacqua et al., 1992). The dissociation of the ribozyme's P1 duplex was also determined to be ~10-fold slower than the dissociation of the model P1 duplex, consistent with the P1 duplex having the same stability on and off the ribozyme. It was suggested that the ribozyme's IGS is less accessible compared to an oligonucleotide in solution (Bevilacqua et al., 1992). Approaches analogous to those presented herein can be used in favorable cases to measure rate constants for dissociation by rapid reaction of oligonucleotide as it dissociates from a preformed duplex.

The method also has limitations. If the inhibitor oligonucleotide has a tendency for self-structure or for aggregation, then measurement of duplex stabilities will be limited to certain ranges of temperature and concentration. This particular limitation also holds for T_m measurements. Indeed, at 30 °C, GGAGGGA appears to exist in multiple conformations, limiting the accessible temperature range for measuring the stabilities of duplexes with this oligonucleotide (data not shown). Compared to the duplex stabilities obtained by T_m methods, the duplex stabilities obtained by substrate inhibition appear to have larger error limits. Nevertheless, ratios

[2] The small differences in the values of $K_d^{IGS'}$ could arise either from systematic differences in the two assays or from a small differential effect of a 5′-phosphate on duplex stability when attached to a C in the ribozyme assay ($^{*pCCCUCUA}_{AGGGAGG}$) vs G in the T1 assay ($^{CCCUCUA}_{AGGGAGGp*}$).

Table 3: Energetic Effects of Single 2′ H Substitutions on Duplex Stability Measured Using the Ribozyme and Ribonuclease T1 Assays[a]

Ribozyme Assay			T1 Assay		
Duplex	$K_d^{IGS'}$(ribozyme)[b] (μM)	K_{rel}(ribozyme)[c]	Duplex	$K_d^{IGS'}$(T1)[d] (μM)	K_{rel}(T1)[e]
5′pCCC UCUA$_5$ AGGGAGG	0.25	**(1)**	5′CCC UCUA AGGGAGGp	0.21	**(1)**
5′pCCCU CUA$_5$ AGGGA**dG**G	0.90	**3.6**	5′CCCU CUA AGGGA**dG**Gp	0.81	**3.8**
5′pCCC UCUA$_5$ AGGG**dA**GG	1.69	**6.8**	5′CCC UCUA AGGG**dA**GGp	1.25	**5.9**
5′pCC CUCUA$_5$ AGG**dG**AGG	0.95	**3.8**	5′CC CUCUA AGG**dG**AGGp	1.05	**5.0**

[a] 50 °C, 50 mM NaMES, pH 6.6, 10 mM $MgCl_2$. [b] $K_d^{IGS'(ribozyme)}$ represents the $K_d^{IGS'}$ value obtained by substrate inhibition using the ribozyme assay. [c] $K_{rel}^{(ribozyme)} = K_d^{IGS'(ribozyme)}$(IGS′ with 2′ H)/$K_d^{IGS'(ribozyme)}$(all-ribose IGS′); $K_{rel} > 1$ indicates a destabilizing effect of the given 2′ H substitution. [d] $K_d^{IGS'(T1)}$ represents the $K_d^{IGS'}$ value obtained by substrate inhibition using the T1 assay. [e] $K_{rel}^{(T1)} = K_d^{IGS'(T1)}$(IGS′ with 2′ H)/$K_d^{IGS'(T1)}$(all-ribose IGS′).

of duplex stabilities obtained using the same stocks of inhibitor give smaller errors (see Materials and Methods and following section). Another limitation is the range of duplexes that can be studied by a particular enzyme assay. However, as demonstrated above with the T1 experiments, the method can be adapted to different enzyme systems. For example, this method was used with an *in vitro* evolved ribozyme that carries out a kinase reaction on a single-stranded RNA oligonucleotide (Lorsch & Szostak, 1995). In principle, this method can be used with any enzymatic (or nonenzymatic) reaction that is specific for single-stranded DNA or RNA (e.g., dimethyl sulfate, 3′−5′ exonuclease, ribonuclease T1), duplexed DNA (e.g., restriction endonucleases), or RNA/DNA hybrids (ribonuclease H). A final disadvantage of this approach is that it is more time-consuming than T_m measurements.

Defining the Energetic Effects of 2′ H Substitutions in a Model P1 Duplex. The effects of 2′ H substitutions on the stability of the P1 duplex were obtained by comparing the stability of model P1 duplexes having 2′ H substitutions with the stability of the normal RNA/RNA P1 duplex (Figure 4A). Based on the reduced stability of purine DNA·pyrimidine RNA duplexes compared to pyrimidine DNA·purine RNA duplexes, it was proposed that single 2′ H substitutions would be more destabilizing in the purine strand (IGS) of the P1 duplex than in the pyrimidine strand (S) (Strobel & Cech, 1993). However, the results here indicate that, with the exception of A24, 2′ H substitutions within IGS′ and S have similar effects on duplex stability (~0.6−0.9 kcal/mol at 50 °C). The larger destabilizing effect at the A24 position is consistent with the greatly reduced stability of duplexes containing dA·rU stretches relative to duplexes containing rA·dT stretches (Martin & Tinoco, 1980), though there is no indication of a differential effect of dA relative to dG in the duplexes with the more random sequences that were used to obtain nearest neighbor rules for DNA/RNA hybrid duplexes (Sugimoto et al., 1995; Freier et al., 1986).

The effects of 2′ H substitutions at C(−2), U(−3), and G22 agree reasonably with the energetic effects obtained from optical melting studies on model P1 duplexes with CCCUCU, with a shorter oligonucleotide, CUCU, or with a modified version of the full-length oligonucleotide, CCCUCC (Figure 4A; Bevilacqua & Turner, 1991; Pyle et al., 1994; Strobel et al., 1994). This suggests that substrate inhibition can detect small differences in duplex stability.

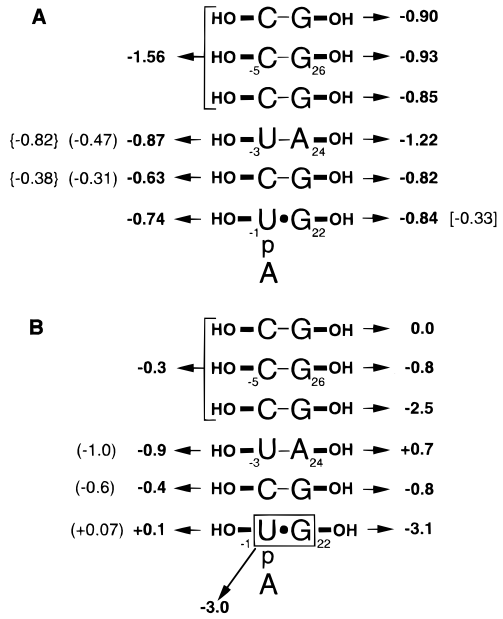


FIGURE 4: The energetic contributions of 2′ OH groups to P1 duplex formation (A) and P1 docking (B), in kcal/mol (50 °C). Also included in (B) is the tertiary stabilization provided by the G·U wobble at the cleavage site (Knitt et al., 1994). The values for duplex effects are calculated from $\Delta\Delta G = -RT \ln(K_d^{IGS',rel})$, where $K_d^{IGS',rel}$ is the effect of 2′ H substitutions on duplex stability (Tables 4 and 7). The values in parentheses are calculated for 50 °C using the thermodynamic parameters, ΔH and ΔS, obtained from previous optical melting studies on model P1 duplexes with CCCUCU [...], CUCU (...), or CCCUCC {...} (Strobel et al., 1994; Bevilacqua & Turner, 1991; Pyle et al., 1994). A negative value of $\Delta\Delta G$ indicates a stabilizing effect of a 2′ OH group relative to a 2′ H group.

Table 4: Tertiary Stabilization from 2′ OH Groups on the Substrate[a]

substrate analog	K_d^{E} [b] (nM)	$K_d^{IGS'}$ [c] (nM)	$K_{tertiary}$ [d]	ΔG_{tert} [e] (kcal/mol)	$\Delta\Delta G_{tert}$ [f] (kcal/mol)
CCCUCUA$_5$	8	250	30	−2.2	**(0)**
CCCUC**d**UA$_5$	22	800	35	−2.3	**+0.1**
CCCU**d**C̄UA$_5$	39	670	16	−1.8	**−0.4**
CCC**d**ŪCUA$_5$	120	970	7	−1.3	**−0.9**
dCdCdCUCUA	240	4920	20	−1.9	**−0.3**

[a] 50 °C, 10 mM $MgCl_2$, 50 mM NaMES, pH 6.6. [b] K_d^{E} is the equilibrium constant for dissociation of S from E·S obtained as described in the Materials and Methods. [c] $K_d^{IGS'}$ is the dissociation constant of the model P1 duplex between S and ^{5}GGAGGGA, measured by substrate inhibition. [d] $K_{tertiary} = [(K_d^{IGS'}/K_d^{E}) - 1]$ and refers to the docking equilibrium (Figure 1, see Materials and Methods). [e] $\Delta G_{tert} = -RT \ln(K_{tertiary})$, $R = 0.00198$ kcal/(mol·K), and $T = 323$ K (50 °C). [f] $\Delta\Delta G_{tert} = \Delta G_{tert}(rS) - \Delta G_{tert}(rS\ analog)$; a negative value indicates that the 2′ H substitution has a destabilizing effect relative to a 2′ OH group.

Quantitating the Tertiary Stabilization Provided by 2′ OH Groups on the Substrate. In previous work, the contributions of individual 2′ OH groups of S to overall binding were obtained from the energetic effects of single 2′ H substitutions (Herschlag et al., 1993). Here, we have dissected the tertiary binding energies provided by the individual 2′ OH groups by comparing the tertiary stability of the normal substrate with the tertiary stabilities of substrates having 2′ H substitutions (Table 4). The tertiary stability of the modified substrates, $K_{tertiary}$, was obtained from comparison of K_d^{E}, the equilibrium constant for dissociation of S from E·S, with $K_d^{IGS'}$, the equilibrium constant for dissociation of S from the corresponding model P1 duplex (Table 4; Figure 1).

The results suggest that the 2′ OH groups at U(−3) and U(−2) contribute 0.9 and 0.4 kcal/mol to tertiary stabilization, respectively (Table 4; Figure 4B, 10 mM $MgCl_2$, 50 °C). In contrast, the 2′ OH groups at C(−6), C (−5), C(−4), and U(−1) do not make significant contributions to tertiary stabilization. Both these results are consistent with previous findings (Figure 4B; Pyle & Cech, 1991; Bevilacqua & Turner, 1991; Herschlag et al., 1993). A previous study determined the effect of single 2′ H substitutions on the tertiary stability of a P1 duplex with a shortened oligonucleotide product, CUCU, using equilibrium dialysis to measure effects on overall binding and optical melting methods to measure the stability of a model P1 duplex (50 mM $MgCl_2$, 15 °C; Bevilacqua & Turner, 1991). Despite the differences in the systems and techniques, the two studies give similar values of tertiary stabilization for the individual 2′ OH groups (Figure 4B).

Specificity of the Binding Site for the 2′ OH at U(−3). In previous work, the specificity of the binding site for the 2′ OH at U(−3) was investigated by substituting the 2′ OH group with a series of functional groups, including a 2′ amino group (Herschlag et al., 1993). The 2′ amino substitution was made in a deoxyribose background (i.e., −3r,dS to −3N,dS). This substitution had inhibitory effects on $(k_{cat}/K_m)^S$, the second-order rate constant for the reaction, $E^G + S \rightarrow$ products, at both pH 5.8 and 7.7. A pK_a of 6.2 was obtained for the 2′ amino group in the dinucleotide 5′-*O*-thymidinyl-3′-*O*-(2′-aminouridyl) phosphate. Hence it was concluded that the binding site for the 2′ substituent at the −3 position discriminates against both an NH_3^+ and an NH_2 group (Herschlag et al., 1993).

Table 5: Energetic Effects of a 2′ Amino Substitution at the −3 Position on P1 Duplex Stability and Docking[a]

substrate	pH	K_d^{E} [b] (nM)	$K_d^{E,rel}$ [c]	$K_d^{IGS'}$ [d] (nM)	$K_d^{IGS',rel}$ [e]	$K_{tertiary}$ [f]
rS	6.6	8	(1)	250	(1)	30
−3N,rS	8.0	85	11	640	1/3	8
−3N,rS	5.0	246	31	7200	1/29	29

[a] 50 °C, 10 mM $MgCl_2$, 50 mM buffer (see Materials and Methods). Binding of rS is pH independent between pH 5 and 8 (G. J. Narlikar and D. Herschlag, unpublished results). In addition, the stability of the model P1 duplex between rS and IGS′ does not change significantly (<3-fold) between pH 5.0 and 8.0. Hence the tertiary stabilization of rS at pH 6.6 can be compared with the tertiary stabilization of −3N,rS at pH 8.0 and 5.0. [b] K_d^{E} is the equilibrium constant for dissociation of S from E·S obtained as described in the Materials and Methods. [c] $K_d^{E,rel} = K_d^{E}(-3N,rS)/K_d^{E}(rS)$; $K_d^{E,rel} > 1$ indicates weaker binding relative to rS. [d] $K_d^{IGS'}$ is the dissociation constant of the model P1 duplex between S and ^{5}GGAGGGA, measured by substrate inhibition. [e] $K_d^{IGS',rel} = K_d^{IGS'}(rS)/K_d^{IGS'}(-3N,rS)$; $K_d^{IGS',rel} < 1$ indicates a destabilizing effect of the 2′ amino substituent on duplex stability. [f] $K_{tertiary} = [(K_d^{IGS'}/K_d^{E}) - 1]$; $K_{tertiary} > 1$ indicates that docking is favored (see Figure 1 and Materials and Methods).

However, with −3N,dS, the inhibitory effect of the 2′ amino group on $(k_{cat}/K_m)^S$ could in principle arise from effects on P1 duplex formation, P1 docking, or the chemical step. It was assumed that the chemical step was unaffected by the 2′ amino substitution at the −3 position (Herschlag et al., 1993), an assumption that has been verified (G. J. Narlikar and D. Herschlag, unpublished results). It was further assumed that the duplex stability would not be substantially changed by the 2′ amino substitution. However, the observation that a run of three 2′ NH_3^+ substituents was highly destabilizing within DNA and RNA duplexes (Aurup et al., 1994) raised questions about this latter assumption. We therefore reexamined the effects of the 2′ amino substituent using a predominantly ribose substrate, −3N,rS, because such substrates allow the effects on duplex formation and tertiary stabilization to be dissected. The results described below indicate that, contrary to the previous conclusion, the 2′ NH_3^+ group at the −3 position stabilizes docking at least as much as a 2′ OH group.

Binding of −3N,rS was measured at pH 8.0 and 5.0 to estimate the contributions of both the 2′ NH_2 and the 2′ NH_3^+ groups. At pH 8.0, −3N,rS binds 11-fold weaker than rS and at pH 5.0, −3N,rS binds 31-fold weaker than rS (Table 5, $K_d^{E,rel}$). However, the destabilizing effect at high pH arises from a destabilizing effect on P1 docking, whereas the destabilizing effect at low pH arises from a large destabilizing effect on P1 duplex formation (Table 5, $K_d^{IGS'}$ and $K_{tertiary}$). This conclusion is described below.

At pH 8.0, the tertiary stability of −3N,rS is the same as that of −3d,rS (Table 6). This indicates that the 2′ NH_2 group does not contribute to tertiary stabilization relative to a 2′ H group. The tertiary stabilization of 0.9 kcal/mol (Table 6) provided by the 2′ amino group at pH 5.0 is a lower limit for the tertiary binding energy provided by the 2′ NH_3^+ group as indicated by the thermodynamic relationships in Scheme 1. In Scheme 1, $(E{\cdot}S^{NH_2})_o$ refers to the open complex and $(E{\cdot}S^{NH_2})_c$ refers to the closed complex (see Figure 1). The stability of the P1 duplex with −3N,rS decreases upon protonation of the 2′ NH_2 group by at least 10-fold (Table 5, $K_d^{IGS'} = 6.4 \times 10^{-7}$ M and 7.2×10^{-6} M at pH 8.0 and 5.0, respectively; Scheme 1, $K_{d'}^{IGS'}/K_d^{IGS'} \geq 10$). Thus, the p$K_a$ of the amino group decreases from 6.2 in the free

Scheme 1

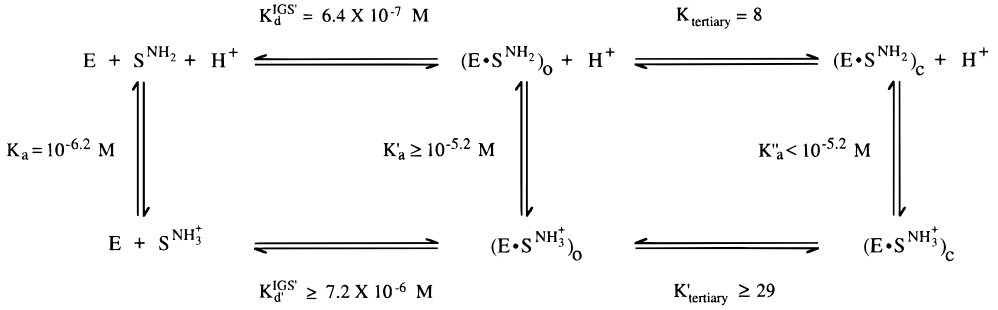


Table 6: Tertiary Stabilization Provided by the 2′ Amino and the 2′ OH Group at the −3 Position

substrate	pH	$K_{tertiary}$[a]	$K_{tertiary}^{rel}$[b]	$\Delta\Delta G_{tert}$[c] (kcal/mol)
−3d,rS	6.6	7	(1)	**(0)**
rS	6.6	30	4.3	**−0.9**
−3N,rS	5.0	29	4.1	**−0.9**[d]
−3N,rS	8.0	8	1.1	**−0.1**

[a] Values of $K_{tertiary}$ are from Tables 4 and 5. [b] $K_{tertiary}^{rel} = K_{tertiary}$(rS or −3N,rS)/$K_{tertiary}$(−3d,rS); $K_{tertiary}^{rel} > 1$ refers to a stabilizing effect on docking relative to −3d,rS. [c] $\Delta\Delta G_{tert} = -RT \ln(K_{tertiary}^{rel})$ at 50 °C; a negative sign indicates a stabilizing effect on docking relative to −3d,rS. [d] The tertiary stabilization provided by the 2′ amino group at pH 5.0 relative to a 2′ H group is a lower limit for the tertiary stabilization provided by the fully protonated 2′ NH_3^+ group (i.e., $\Delta\Delta G_{tert} \leq -0.9$ kcal/mol; Scheme 1 in text).

substrate ($S^{NH_3^+} \rightleftharpoons S^{NH_2} + H^+$; $K_a = 10^{-6.2}$ M) to 5.2 or below in the P1 duplex [$(E \cdot S^{NH_3^+})_o \rightleftharpoons (E \cdot S^{NH_2})_o + H^+$; $K'_a \geq 10^{-5.2}$ M]. Hence, at pH 5.0, the 2′ amino group is not fully protonated in the P1 duplex so that its observed tertiary contribution relative to a 2′ H group is a lower limit for the tertiary contribution of the fully protonated 2′ NH_3^+ species.

Quantitating the Tertiary Stabilization Provided by 2′ OH Groups on the IGS. In previous work, the binding energies provided by individual 2′ OH groups on the IGS were obtained from comparisons of the binding affinity of rP for the normal ribozyme with its affinity for modified ribozymes having single 2′ H substitutions in the IGS (Strobel & Cech, 1993). Effects on P1 duplex stability were distinguished from effects on P1 docking via an indirect approach using 2′ OCH_3 substitutions. Unlike a 2′ H group, a 2′ OCH_3 group prefers the same sugar conformation as preferred by a 2′ OH group and does not affect the stability of an RNA/RNA duplex (Uesugi et al., 1979; Guschlbauer & Jankowski, 1980; Herschlag et al., 1993). Hence, the ability of a 2′ OCH_3 substitution to rescue the effect of a 2′ H substitution was interpreted to mean that the 2′ H substitution affected solely duplex stability. The average destabilizing effect of a 2′ H substitution on duplex stability was thereby estimated to be ~6-fold (1.1 kcal/mol, 42 °C; Strobel & Cech, 1993).

Here, we have used substrate inhibition to directly measure the effects of single 2′ H substitutions on the stability of a model P1 duplex (Table 7, $\Delta\Delta G_{tert}$). There is a large destabilizing effect of ~7-fold at A24, but smaller effects of 4-fold at other positions (Table 7). The tertiary stabilization provided by the modified ribozymes, $K_{tertiary}$, was obtained from comparison of K_d^E, the equilibrium constant for dissociation of rP from E·rP (Strobel & Cech, 1993), with $K_d^{IGS'}$, the equilibrium constant for dissociation of rP from the corresponding model P1 duplex (Table 7; Figure 1). The 2′ OH groups of G22 and G25 provide significant tertiary stabilization, as previously concluded (Table 7, −3.1 and −2.5 kcal/mol, respectively; Strobel & Cech, 1993). In addition, separation of the duplex effects of 2′ H substitutions has revealed that the 2′ OH groups of G23 and G26 provide small amounts of tertiary stabilization (−0.8 kcal/mol each, Table 7) whereas the 2′ OH group of A24 has a small destabilizing effect on P1 docking (+0.7 kcal/mol, Table 7).

DISCUSSION

Binding of the *Tetrahymena* ribozyme's oligonucleotide substrate involves P1 duplex formation with the ribozyme's IGS followed by docking of the P1 duplex into tertiary interactions (Figure 1). Based on previous work and the results here, we have quantitated the tertiary binding energy provided by individual 2′ OH groups on the P1 duplex (Figure 4B). The energetic effects of specific 2′ OH groups are discussed below.

It is suggested that the energy from tertiary binding interactions of groups on the P1 duplex is used for catalysis. This extends the catalytic principle of "intrinsic binding energy" originally proposed for protein enzymes to an RNA enzyme.

A 2′ NH_3^+ Group Can Substitute for the 2′ OH at the −3 Position. It was previously concluded that the binding site for the 2′ substituent at the −3 position is highly specific for a 2′ OH group, based in part on the inability of a 2′ NH_2 or a 2′ NH_3^+ group to substitute for the 2′ OH group in a DNA background (Herschlag et al., 1993). However, the results here indicate that the inability of the 2′ NH_3^+ group to substitute for the 2′ OH group arose from its large destabilizing effect on P1 duplex stability. The 2′ NH_3^+ group stabilizes P1 docking to at least the same extent as a 2′ OH group (Tables 5 and 6). Hence, the previous interpretation of the 2′ NH_3^+ result was incorrect. The ability of a 2′ NH_3^+ to replace the OH group most simply suggests the absence of a hydrogen bond donor or metal ion on the ribozyme at this position. The inability of 2′ NH_2, OCH_3, and F to replace the OH or NH_3^+ group (Table 5; Pyle et al., 1992; Herschlag et al., 1993; D. Herschlag, unpublished results) also provides no evidence for an interaction with a hydrogen bond donor or metal ion on the ribozyme. Analysis of a series of ribozyme mutants has led to the suggestion that N1 of residue A302 within the J8/7 region accepts a hydrogen bond from the 2′ OH group (Pyle et al., 1992). Though more complex models and additional interactions are possible, all of the data are consistent with a simple model in which the 2′ OH at position −3 donates a hydrogen bond to a group on the ribozyme.

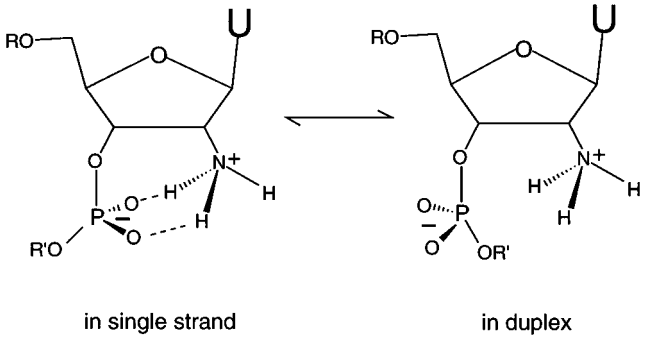
An additional question raised from these results is: How does a single 2′ NH_3^+ substituent have such a large effect on duplex stability (≥2.1 kcal/mol, 50 °C)? Substitution of three 2′ NH_3^+ groups was greatly destabilizing within

Table 7: Contributions of 2′ OH Groups on the IGS to P1 Duplex Stability and Docking[a]

P1 Duplex	K_d^E[b] (nM)	$K_d^{IGS'}$[c] (nM)	$K_d^{IGS',rel}$[d]	$K_{tertiary}$[e]	ΔG_{tert}[f] (kcal/mol)	$\Delta\Delta G_{tert}$[g] (kcal/mol)
5′CCC UCU AGGGAGG	2.5	1500	(1)	600	-4.1	**(0)**
5′CCC UC U AGGGAG**dG**	930 [2400]	5600	3.7	5.0 [1.3]	-1.0 [-0.2]	**-3.1** **[-3.9]**
5′CCCU CU AGGGA**dG**G	30	5400	3.6	179	-3.3	**-0.8**
5′CCC UCU AGGG**dA**GG	5.8	10200	6.8	1760	-4.8	**+0.7**
5′CC CUCU AGG**dG**AGG	430	5700	3.8	12	-1.6	**-2.5**
5′C CCUCU AG**dG**GAGG	38	6500	4.3	170	-3.3	**-0.8**
5′CCC UCU A**dG**GGAGG	10	6200	4.1	619	-4.1	**0.0**

[a] 50 °C, 10 mM $MgCl_2$. [b] K_d^E is the equilibrium constant for dissociation of rP from the E·rP complex and is extrapolated to 50 °C from the values in Strobel and Cech (1993) as described in the Materials and Methods. The values in brackets are from an independent set of measurements by Strobel and Cech (1993). [c] $K_d^{IGS'}$ is the dissociation constant of the model P1 duplex between rP and 5′GGAGGGA obtained as described in Materials and Methods. [d] $K_d^{IGS',rel} = K_d^{IGS'}$(IGS′ with 2′ H)/$K_d^{IGS'}$(all-ribose IGS′); $K_d^{IGS',rel} > 1$ indicates a destabilizing effect of the given 2′ H substitution. [e] $K_{tertiary}$ refers to the docking equilibrium (Figure 1, see Materials and Methods). [f] $\Delta G_{tert} = -RT\ln(K_{tertiary})$, $R = 0.00198$ kcal/(mol·K), and $T = 323$ K (50 °C). [g] $\Delta\Delta G_{tert} = \Delta G_{tert}$(normal ribozyme) − ΔG_{tert}(ribozyme with 2′ H); a negative value indicates that the 2′ H substitution has a destabilizing effect relative to a 2′ OH group.

Scheme 2



both RNA and DNA duplexes, providing no indication of a dependence on duplex geometry (Aurup et al., 1994). A model that can account for the 2′ NH_3^+ effect is presented in Scheme 2. It is suggested that the apparent weakening of the duplex arises not from an effect within the duplex, but rather from stabilization of a conformation of the single-stranded oligonucleotide that is incompatible with duplex formation (Scheme 2). Specifically, the 2′ NH_3^+ group could donate hydrogen bonds to one or both of the neighboring 3′ nonbridging phosphoryl oxygens. Though, like a 2′ NH_3^+ group, a 2′ OH and NH_2 group can also form one or two intramolecular hydrogen bonds, both are uncharged and are weaker hydrogen bond donors than NH_3^+. It may be possible to use the instability of duplexes upon protonation of a 2′ amino substituent to trigger local conformational rearrangements or cause local unfolding of nucleic acids in a spatially and temporally controlled manner.

An Inhibitory Effect of a 2′ OH Group. A shortened substrate, $UCUA_5$, shows reduced tertiary stabilization compared to the full-length substrate (G. J. Narlikar, M. Khosla, and D. Herschlag, unpublished results). The P1 duplex with $UCUA_5$ has all the 2′ OH groups implicated in tertiary stabilization, but is less rigid than the normal P1 duplex. This suggests that the rigidity of the P1 duplex is necessary to make strong tertiary interactions. However, the rigidity of the duplex may also place constraints on docking that result in unfavorable interactions. The small destabilizing effect of the 2′ OH of A24 relative to a 2′ H group (+0.7 kcal/mol) is consistent with such an effect. This destabilization could arise from direct interactions with groups on the ribozyme or indirectly, from constraints placed on the sugar conformation by the 2′ OH group. A deoxyribose sugar, which lacks the 2′ substituent and therefore has a greater degree of conformational freedom compared to a ribose sugar, may facilitate rearrangements that avoid the unfavorable interactions (Saenger, 1988).

Reevaluating the Interpretation of the Energetic Effects of the 2′ OH Group at G22. Based on the energetic effects of modified ribozymes, Strobel and Cech (1993) proposed that the 2′ OH group of G22 of the ribozyme's IGS acts as a molecular linchpin that mediates recognition of the P1 duplex by both the J8/7 and J4/5 regions of the catalytic core (Strobel & Cech, 1993). Here we propose an alternative and more general explanation to account for these energetic effects. This reinterpretation in no way contradicts the structural models and experimental data that suggest that J8/7 and J4/5 are both in proximity to the docked P1 duplex (Michel & Westhof, 1990; Pyle et al., 1992; Wang & Cech, 1992; Wang et al., 1993).

Strobel and Cech (1993) showed that replacing the 2′ OH group of G22 to give the dG22 ribozyme resulted in 370−950-fold weaker binding of rP [Tables I and II from Strobel and Cech (1993)]. In the context of the dG22 ribozyme, the 2′ OH at U(−3) no longer contributes significantly to rP binding. The dG22 modification also affects G binding. It has been shown for the normal ribozyme that binding of rS

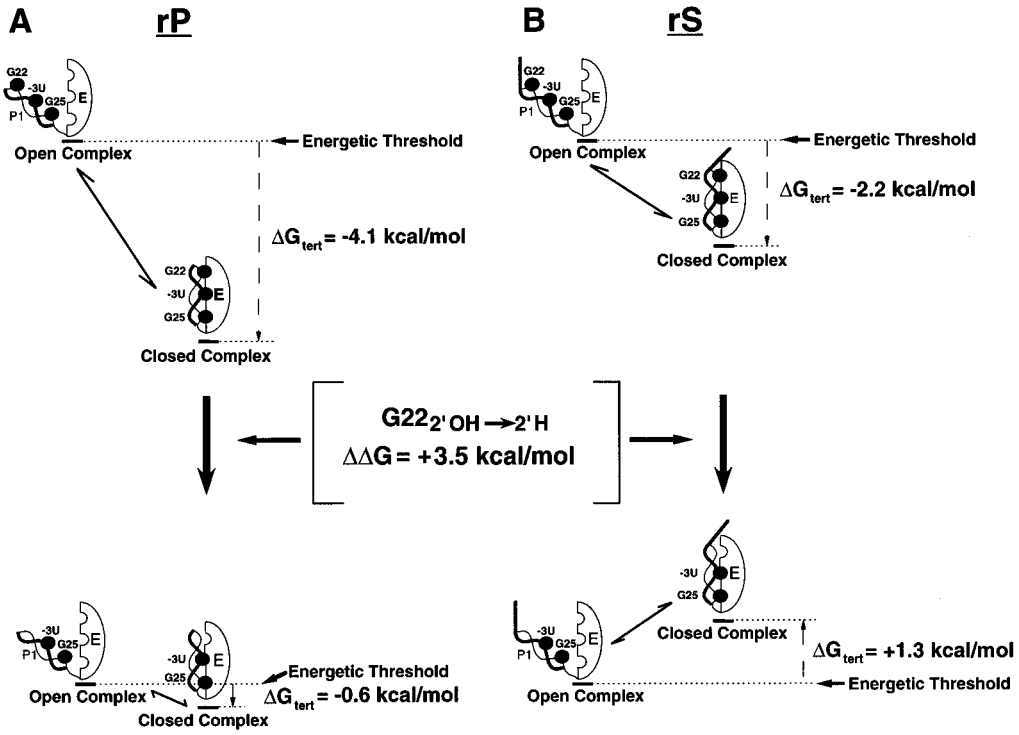


FIGURE 5: Threshold model for oligonucleotide binding. Tertiary interactions stabilize the closed complexes with rP (A) and rS (B) relative to their respective open complexes by 4.1 and 2.2 kcal/mol [50 °C, ΔG_{tert}; Tables 4 and 7 (Narlikar et al., 1995)]. rP and rS are shown as thick lines. The 2′ OH groups of G22, G25, and U(−3), which provide the largest contributions to tertiary stabilization, are shown schematically as filled circles. Binding in the closed complex is maintained if the loss in tertiary stabilization is less than the energetic difference between the open and closed complexes (4.1 kcal/mol for rP and 2.2 kcal/mol for rS). Loss of any tertiary interaction contributing more than the energetic difference between the open and closed complexes destabilizes the closed complex beyond the "energetic threshold" separating the open and closed complexes. This results in predominant binding in the open complex. (A) The energetic consequences of a 2′ H substitution at G22 on rP binding and (B) on rS binding. The 2′ OH of G22 contributes ~3.5 kcal/mol to tertiary stabilization.[3] Hence loss of this tertiary stabilization is predicted to destabilize the closed complex with rP, such that it is comparable in energy to the open complex [$\Delta G_{tert} = -4.1 - (-3.5) = -0.6$ kcal/mol]. Loss of any further tertiary interaction will result in at most a 3-fold effect on tertiary binding. With rS, loss of the tertiary stabilization provided by the 2′ OH at G22 destabilizes the closed complex such that it is 1.4 kcal/mol less stable than the open complex [$\Delta G_{tert} = -2.2 - (-3.5) = +1.3$ kcal/mol]. As a result, rS binds predominantly in the open complex. The experiments by Strobel and Cech (1993) were carried out at 30 and 42 °C. The change in tertiary stability over the range of 30 to 50 °C is small (Narlikar & Herschlag, 1996), so that none of the conclusions of the above analysis of binding at 50 °C are affected.

stabilizes G binding by ~1 kcal/mol and vice versa (McConnell et al., 1993). Replacing the 2′ OH at G22 with a 2′ H abolished energetic coupling between rS and G binding. Hence it was suggested that the 2′ OH group at G22 mediates recognition of the P1 duplex by both J8/7, which is in proximity to the 2′ OH group of U(−3), and J4/5, which is in proximity to bound G (Michel & Westhof, 1990; Pyle et al., 1992; Wang & Cech, 1992; Wang et al., 1993).

We propose a model in which the energetic effects described above arise from loss of *any* tertiary interaction that results in destabilization of the docked P1 duplex beyond the energetic "threshold" separating the open and closed complexes (Figure 5). Tertiary interactions stabilize the closed complex with rP by 4.1 kcal/mol relative to the open complex [Figure 5A (Narlikar et al., 1995)]. Loss of the 3.5 kcal/mol of tertiary stabilization provided by the 2′ OH at G22 is predicted to make the closed complex comparable in energy to the open complex (Figure 5A).[3] Further loss of a tertiary interaction, such as the 2′ OH of −3, will therefore have at most a small destabilizing effect on overall binding, consistent with the observations of Strobel and Cech (1993). For rS, the tertiary stabilization is less than for rP ($\Delta G_{tert} = -2.2$ vs -4.1 kcal/mol, respectively; Narlikar et al., 1995). Hence with rS, the dG22 modification is predicted to result in a closed complex that it is less stable than the open complex. As a result, rS binds predominantly in the open complex (Figure 5B). The observed absence of coupling between rS and G with the dG22 ribozyme is then expected because substrates bound in the open complex do not show coupled binding with G (McConnell et al., 1993).

Other previous observations can also be explained by this "threshold model". These are not accounted for by the previous "linchpin" model. Substituting U(−1) with a C destabilizes tertiary binding by 3.0 kcal/mol due to disruption of the tertiary stabilization provided by the G·U wobble at the cleavage site (Knitt et al., 1994; Pyle et al., 1994). This destabilization is greater than the energetic threshold of 2.2 kcal/mol separating the open and closed complexes with rS. As predicted by the above model, the C(−1) substitution has energetic effects analogous to those observed with the dG22 modification: loss of coupled binding with G and absence of further destabilization upon replacing the 2′ OH at U(−3) with a 2′ H (Knitt et al., 1994; G. J. Narlikar and D. Herschlag, unpublished results).[4] The energetic effects of a series of base-pair substitutions at the cleavage site are also consistent with the above model (Strobel & Cech, 1995, 1996). In summary, the threshold model presented here

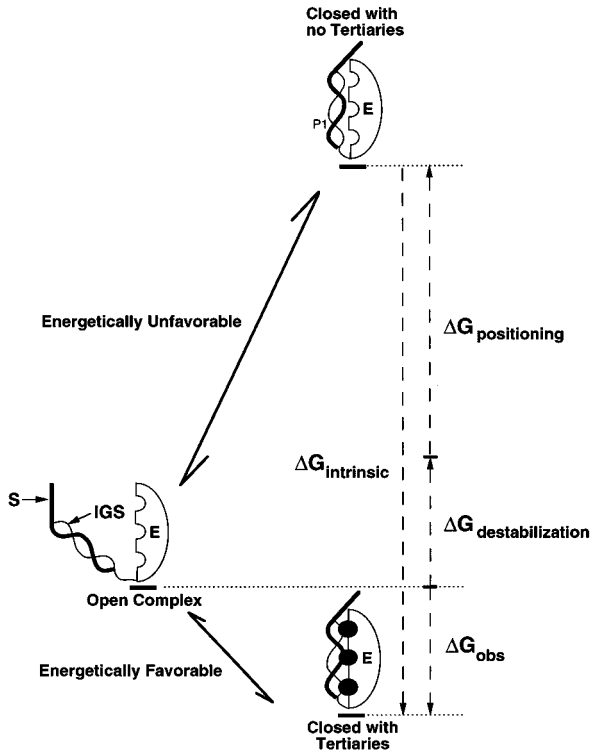
[3] The average of the two $\Delta\Delta G_{tert}$ values in Table 7 is used in the analysis of Figure 5.

provides a framework to describe the observed energetic effects of substituting functional groups on the P1 duplex. Such a model may also explain analogous effects in other biological systems.

Intrinsic Binding Energy of Groups on the P1 Duplex Is Used for Catalysis. The sum of the tertiary stabilization provided by individual groups on the P1 duplex is 11.0 kcal/mol (Figure 4B). This is much larger than the observed tertiary stabilization of rS, which is only 2.2 kcal/mol (Table 4). One explanation for this discrepancy is that the binding energies of the individual tertiary interactions are not additive. If this were the case, concomitant removal of multiple tertiary binding interactions would result in a maximal loss of 2.2 kcal/mol of tertiary binding energy. However, removal of the binding interactions provided by the G·U wobble pair and the 2′ OH groups of U(−3) and G22 results in a loss of 6 kcal/mol of tertiary binding energy (G. J. Narlikar and D. Herschlag, unpublished results). To account for this substantially larger tertiary binding energy compared to the observed binding energy, we propose a model in which there is an energetic cost for docking of rS that is paid for by the intrinsic binding energy of tertiary interactions (Figure 6).[5] The observed tertiary stabilization of 2.2 kcal/mol is what remains after the intrinsic binding energy of tertiary interactions is utilized to overcome this energetic barrier.

Jencks has proposed that protein enzymes use the intrinsic binding energy of groups away from the site of chemical cleavage for catalysis (Jencks, 1975). If the *Tetrahymena* ribozyme uses the same catalytic principle, then a decrease in reactivity is predicted when the binding energy of tertiary interactions is insufficient to overcome the energetic barrier for docking. As expected, a reduction in reactivity is observed with the dG22 modification and a 2′ OCH_3 substitution at the −3 position of S, both of which result in large decreases in tertiary binding energy (Table 7; Strobel & Cech, 1993, Narlikar et al., 1995; Narlikar & Herschlag, 1996).

What are the mechanisms by which intrinsic binding energy is utilized for catalysis in the *Tetrahymena* ribozyme? This question is addressed in part by recent work that suggests that the intrinsic binding energy of substituents on the P1 duplex is used to pay for the cost of substrate destabilization and the cost of entropic fixation within the closed complex (Narlikar et al., 1995). It was suggested that 2.3 kcal/mol of the intrinsic binding energy is used to position rS in an electrostatic environment that is better suited to stabilizing the transition state (Figure 6, $\Delta G_{destabilization}$). This combination of substrate destabilization and transition state stabilization, in principle, accounts for 10^4-fold of the 10^{11}-fold catalysis observed with the *Tetrahymena* ribozyme (Narlikar et al., 1995). Part of the intrinsic binding energy may also used for positioning rS relative to G within the active site, thereby reducing the entropic barrier for reaction (Figure 6, $\Delta G_{positioning}$). The large effective concentration of 2200 M obtained for the 3′ OH functional group of rP suggests that the *Tetrahymena* ribozyme is capable of precise positioning (Narlikar et al., 1995). The intrinsic binding energy used for inducing substrate destabilization (2.3 kcal/mol) and the expressed binding energy (2.2 kcal/mol) together account for 4.5 kcal/mol of the ≥6 kcal/mol of intrinsic binding energy. This suggests that at least 1.5 kcal/mol of binding energy is used for positioning the substrate within the active site.[5] Positioning can also be achieved by interactions within the folded ribozyme that help align the IGS with respect to the active site. Hence, part of the cost of positioning can also be paid for by the interactions involved in folding. Indeed, previous work has provided



$$\Delta G_{obs} = \Delta G_{intrinsic} - (\Delta G_{destabilization} + \Delta G_{positioning})$$

FIGURE 6: Model for the use of intrinsic binding energy for positioning and substrate destabilization. In the open complex, S is localized to the ribozyme simply by base-pairing interactions with the IGS. In the closed complex, tertiary interactions position the P1 duplex within the active site. Groups involved in tertiary interactions are shown schematically as filled circles. In the absence of tertiary interactions, the closed complex is destabilized relative to the open complex, because of a destabilizing interaction between the bridge oxygen of the reactive phosphoryl group and a Mg ion that becomes stabilizing in the transition state and because of entropic fixation of the substrate within the active site ($\Delta G_{destabilization}$ and $\Delta G_{positioning}$; Narlikar et al., 1995). The intrinsic binding energy, $\Delta G_{intrinsic}$, of tertiary interactions is used to pay for overcoming these destabilizing effects. ΔG_{obs} (=2.2 kcal/mol, 50 °C) is the observed tertiary stabilization of the substrate after part of the intrinsic binding energy has been used to pay for substrate destabilization and entropic fixation. The energetic cost of electrostatic destabilization equals 2.3 kcal/mol (Narlikar et al., 1995). The energetic cost of positioning cannot be estimated because the extent of independence of tertiary binding interactions has not been established (see text).

[4] In contrast to rS, rP, which has greater tertiary stabilization, is predicted to still be docked with the C(−1) substitution. As expected, replacing the 2′ OH at U(−3) with a 2′ H results in a 3−4-fold destabilizing effect on the tertiary binding of CCCUCC (Strobel & Cech, 1995).

[5] In the above definition of intrinsic binding energy, a DNA/DNA P1 duplex having a G·C pair at the cleavage site is defined as having zero intrinsic binding energy (Figure 6, closed complex with no tertiaries). This reference state arises from attributing tertiary binding energies to the 2′ OH groups and the G·U wobble at the cleavage site by replacing them with 2′ H groups and a G·C Watson−Crick pair, respectively. This is analogous to the approach of attributing binding energies to functional groups in protein side chains by mutating them to side chains lacking the functional groups (Fersht, 1987). Nevertheless, in addition to removing interactions made by a 2′ OH group in the docked state, a 2′ H group may make interactions that are unfavorable relative to the corresponding interactions in solution.

evidence that the J1/2 region, which joins the IGS to the catalytic core, helps position the P1 duplex relative to the catalytic core, presumably reducing the entropic barrier for making tertiary binding interactions (Herschlag, 1992).

A number of protein enzymes have been suggested to use binding energy for catalysis (Jencks, 1975). Extensive site directed mutagenesis has identified amino acid residues in tyrosyl-tRNA synthetase that do not contribute significantly to the observed binding energy of the substrates. Instead, their binding energy is utilized to accelerate the formation of tyrosyl-AMP (Fersht, 1987). In isoleucyl-tRNA synthetase, the binding energy of the amino and carboxyl groups appears to be used for carrying out a conformational change that aligns the substrates within the active site (Holler et al., 1973). The binding energy of the amino acid side chains of chymotrypsin's polypeptide substrate is not manifested in the observed K_m values. Rather, the binding energy of these groups contributes to k_{cat} (Baumann et al., 1970). It has been suggested that the binding energy of the groups on the substrate is used for positioning the carbonyl group of the reactive peptide bond within the oxyanion hole of the active site (Henderson, 1970; Robertus et al., 1972; Jencks, 1975).

Jencks has proposed that the use of intrinsic binding energy is a fundamental feature of catalysis by protein enzymes. The results presented here and previously (Narlikar et al., 1995) with the *Tetrahymena* ribozyme extend this concept to RNA enzymes, thereby underscoring the generality of using binding energy for biological catalysis. RNA enzymes may prove to be generally tractable for energetic analyses of macromolecular catalysis. In the *Tetrahymena* ribozyme, a substrate that is precisely positioned within the active site can be distinguished energetically and structurally from a substrate that is simply localized to the ribozyme but not precisely positioned (closed vs open complex, Figure 1; Bevilacqua et al., 1992; Wang et al., 1993; Narlikar & Herschlag, 1996). Thus, the use of intrinsic binding energy can be directly correlated with a distinct conformational change that leads to catalysis.

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SUPPORTING INFORMATION AVAILABLE

One table comparing the energetic effects of base-pair substitutions in the context of different duplexes. The duplex stabilities were obtained by substrate inhibition, nearest neighbor calculations, and T_m measurements (2 pages). Ordering information is given on any current masthead page.

REFERENCES

Aurup, H., Tuschl, T., Benseler, F., Ludwig, J., & Eckstein, F. (1994) *Nucleic Acids Res. 22*, 20−24.

Baumann, W. K., Bizzozero, S. A., & Dutler, H. (1973) *Eur. J. Biochem. 39*, 381−391.

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.

Bevilacqua, P. C., Li, Y., & Turner, D. H. (1994) *Biochemistry 33*, 11340−11348.

Fersht, A. R. (1987) *Biochemistry 26*, 8031−8037.

Freier, S. M., Kierzek, R., Jaeger, J. A., Sugimoto, N., Caruthers, M., Nielson, T., & Turner, D. H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 9373−9377.

Guschlbauer, W., & Jankowski, K. (1980) *Nucleic Acids Res. 8*, 1421−1433.

Henderson, R. (1970) *J. Mol. Biol. 54*, 341−354.

Herschlag, D. (1992) *Biochemistry 31*, 1386−1399.

Herschlag, D., & Cech, T. R. (1990) *Biochemistry 29*, 10159−10171.

Herschlag, D., Eckstein, F., & Cech, T. R. (1993) *Biochemistry 32*, 8299−8321.

Holler, E., Rainey, P., Orme, A., Bennett, E. L., & Calvin, M. (1973) *Biochemistry 12*, 1150−1159.

Jencks, W. P. (1975) *Adv. Enzymol. 43*, 219−410.

Knitt, D. S., & Herschlag, D. (1996) *Biochemistry 35*, 1560−1570.

Knitt, D. S., Narlikar, G. J., & Herschlag, D. (1994) *Biochemistry 33*, 13864−13879.

Li, Y., Bevilacqua, P. C., Mathews, D., & Turner, D. H. (1995) *Biochemistry 34*, 14394−14399.

Lorsch, J. R., & Szostak, J. W. (1995) *Biochemistry 34*, 15315−15327.

Martin, F. H., & Tinoco, I. J. (1980) *Nucleic Acids Res. 8*, 2295−2299.

McConnell, T. S., Cech, T. R., & Herschlag, D. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 8362−8366.

Michel, F., & Westhof, E. (1990) *J. Mol. Biol. 216*, 585−610.

Narlikar, G. J., & Herschlag, D. (1996) *Nature, Struct. Biol. 3*, 701−710.

Narlikar, G. J., Gopalakrishnan, V., McConnell, T. S., Usman, N., & Herschlag, D. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 3668−3672.

Pyle, A. M., & Cech, T. R. (1991) *Nature 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.

Pyle, A. M., Moran, S., Strobel, S. A., Chapman, T., Turner, D. H., & Cech, T. R. (1994) *Biochemistry 33*, 13856−13863.

Robertus, J. D., Kraut, J., Alden, R. A., & Birktoft, J. J. (1972) *Biochemistry 11*, 4293−4303.

Saenger, W. (1988) *Principles of nucleic acid structure*, pp 331−349, Springer-Verlag, New York.

Serra, M. J., & Turner, D. H. (1995) *Methods Enzymol. 259*, 242−261.

Strobel, S. A., & Cech, T. R. (1993) *Biochemistry 32*, 13593−13604.

Strobel, S. A., & Cech, T. R. (1994) *Nat. Struct. Biol. 1*, 13−17.

Strobel, S. A., & Cech, T. R. (1995) *Science 267*, 675−679.

Strobel, S. A., & Cech, T. R. (1996) *Biochemistry 35*, 1201−1211.

Strobel, S. A., Cech, T. R., Usman, N., & Beigelman, L. (1994) *Biochemistry 33*, 13824−13835.

Sugimoto, N., Sasaki, M., Kierzek, R., & Turner, D. H. (1989) *Chem. Lett.*, 2223−2226.

Sugimoto, N., Nakano, S., Katoh, M., Matsumura, A., Nakamuta, H., Ohmichi, T., Yoneyama, M., & Sasaki, M. (1995) *Biochemistry 34*, 11211−11216.

Uchida, T., & Egami, F. (1971) *The Enzymes*, 3rd ed., Vol. 4, pp 205−250, Academic Press, New York.

Uesugi, S., Miki, H., Ikehara, M., Iwahashi, H., & Kyogoku, Y. (1979) *Tetrahedron Lett. 42*, 4073−4076.

Wang, J.-F., & Cech, T. R. (1992) *Science 256*, 526−529.

Wang, J.-F., Downs, W. D., & Cech, T. R. (1993) *Science 260*, 504−508.

Zaug, A. J., Grosshans, C. A., & Cech, T. R. (1988) *Biochemistry 27*, 8924−8931.

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