

## Direct Demonstration of the Catalytic Role of Binding Interactions in an Enzymatic Reaction<sup>†</sup>

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**ABSTRACT:** It has been suggested that the fundamental feature that distinguishes enzymes from simple chemical catalysts is the ability of enzymes to use binding interactions for catalysis. Results with the *Tetrahymena* group I RNA enzyme described herein directly demonstrate the catalytic contributions of binding interactions. With wild-type ribozyme, specific functional groups at a distance from the site of chemical transformation facilitate substrate binding without accelerating reaction of bound substrate; with modified ribozymes, these functional groups provide the same overall energetic effect but instead accelerate reaction of bound substrate without increasing binding. These observations are quantitatively described by a structural framework that was established by previous results. The P1 duplex between the substrate and the ribozyme's recognition sequence exists in two states, the open complex, in which the substrate is localized to the ribozyme solely by base-pairing interactions, or the closed complex, in which the duplex is docked into tertiary interactions and positioned with respect to the catalytic groups in the active site. In the absence of sufficient binding energy to ensure stable docking in the ground state, added P1 functional groups accelerate reaction of the bound substrate by helping to overcome the energetic barrier for docking into the reactive, closed complex. When the functional groups present on the P1 duplex are sufficient to ensure stable docking in the closed complex, added functional groups give stronger binding without accelerating reaction of the bound substrate. This behavior is a manifestation of the inextricable link between binding interactions and catalysis. The conclusions also have implications for interpreting the effects of site-directed mutagenesis and for the evolution of active site interactions.

Catalysis is defined as stabilization of a reaction's transition state without equivalent stabilization of the ground state (1–3). Simple chemical catalysts such as general acids and bases, metal ions, and hydrogen bonding and charged molecules specifically stabilize a reaction's transition state by exploiting differences between the ground state and transition state. Enzymes use analogous functional groups that carry out general acid–base, metal ion, and electrostatic catalysis, yet the rate enhancements achieved by enzymes are typically much greater than those achieved by simple chemical catalysts.

What distinguishes enzymes from simple, small molecule catalysts? Jencks proposed that the fundamental distinction is the ability of enzymes to use binding interactions away from the site of bond cleavage for catalysis (4). This conclusion was based on a comprehensive analysis that included several examples in which the addition of a substrate

group increased  $k_{\text{cat}}$  instead of increasing substrate binding, even though the added group was distant from the site of chemical transformation (e.g., see refs 5–9). Subsequently, analogous results were obtained from site-directed mutagenesis and substrate modifications in additional enzymes (e.g., refs 10–13). Recent results with the hammerhead and *Tetrahymena* ribozymes have extended this principle to RNA enzymes (14–17). As noted by Jencks and others, binding interactions can facilitate reactions by positioning the substrates with respect to one another and with respect to catalytic groups (4, 11, 18–24). Binding interactions can also accelerate reactions by desolvating and electrostatically or geometrically destabilizing substrates (for reviews, see refs 4 and 25). This has led to the view that the catalytic contributions of functional groups involved in substrate binding and conventional catalytic groups such as general acids, general bases, and metal ions are inextricably linked (for reviews, see refs 4 and 18).

Albery and Knowles presented a distinct analysis of enzymatic catalysis that separated the contributions from substrate binding groups and conventional catalytic groups (26). They noted that the energetic contributions of enzymatic functional groups can be separated into two limiting classes, uniform binding and specific transition state stabi-

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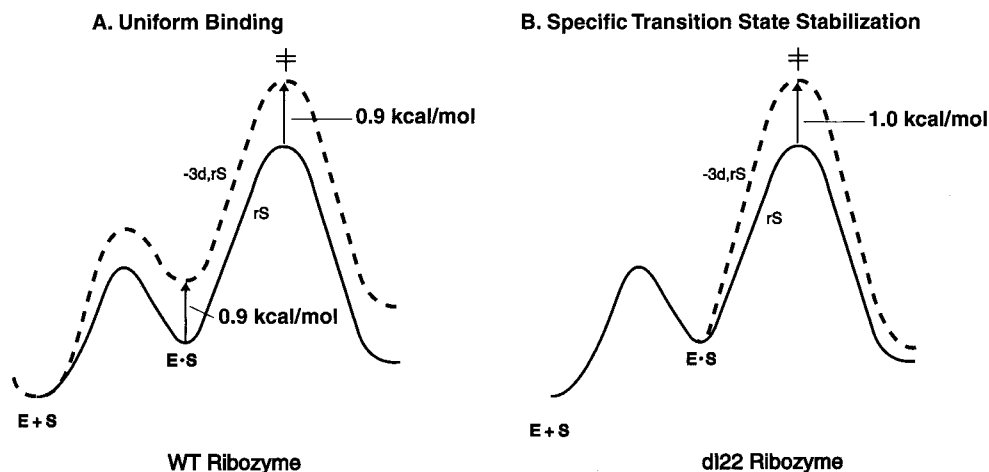


FIGURE 1: Energetic effect of removing the 2'-OH group at position  $-3$  of S in the context of two different ribozymes. Schematic free energy profiles for catalysis by the WT ribozyme (A) and dl22 ribozyme (B). The dl22 ribozyme lacks two groups that make tertiary interactions, the 2'-OH group of G22 and the exocyclic amino group of G22 (Figure 2B). Reaction profiles for  $-3d,rS$  and  $rS$  are shown by dashed and solid lines, respectively, and are drawn for conditions of subsaturating E. E·S refers generically to the enzyme·substrate complex and ‡ to the transition state for the chemical reaction. Guanosine binding is included in the chemical step (not shown; see Materials and Methods). The arrows indicate the unfavorable effect from removal of the ( $-3U$ ) 2'-OH group for the WT ribozyme (A) and for the dl22 ribozyme (B). The energetic effects on binding ( $\Delta\Delta G_{\text{bind}}$ ) and the chemical step ( $\Delta\Delta G^{\ddagger}$ ) were obtained as described in Materials and Methods.

lization (see Figure 1 and below). Uniform binding increases the binding of substrate but does not enhance reaction of bound substrate. In contrast, specific transition state stabilization has no effect on binding but increases the rate of reaction of bound substrate. Uniform binding groups were described as interacting with substrate functional groups that do not undergo charge rearrangements during the course of the reaction. Groups that specifically stabilize the transition state were described as recognizing the structural and electronic changes associated with bond cleavage and bond formation. It was also noted that certain residues could provide a hybrid effect, providing some stabilization of the ground state but greater stabilization in the transition state. Thus, a continuum of energetic effects is possible for different residues. However, the treatment implied that the respective energetic contribution of each residue to binding and transition state stabilization remains fixed, with the respective energetic contributions defining the fundamental catalytic role of the residue.

The results herein reconcile these distinct views. We have observed that a specific functional group on the substrate of the *Tetrahymena* ribozyme that contributes uniform binding in the context of the wild-type ribozyme instead provides specific transition state stabilization in the context of a modified ribozyme. These and related observations described here directly demonstrate the catalytic role of binding interactions.

## MATERIALS AND METHODS

**Ribozymes and Oligonucleotide Substrates.** The L-21 *ScaI* (WT) ribozyme was prepared via *in vitro* transcription (27). Modified ribozymes were prepared by ligation of a synthetic 17-base oligonucleotide to a shortened RNA transcript (L-38 *ScaI*) using T4 DNA ligase and a DNA splint as described previously (28, 29). For each modified ribozyme, the fraction of ligated ribozyme was quantitated using primer extension via reverse transcription (29) and also by using T4 polynucleotide kinase, [ $\gamma$ - $^{32}\text{P}$ ]ATP, and ADP to quanti-

tatively exchange the 5'-phosphate on the L-38 transcript with [ $^{32}\text{P}$ ]phosphate and simultaneously label the 5'-hydroxy terminus of the ligated ribozymes (30). The two approaches gave the same results within 10%. The unligated transcript does not interfere with the kinetic assays because it lacks the internal guide sequence and hence cannot bind or cleave the oligonucleotide substrate (29). Oligonucleotides were synthesized using standard solid-phase synthesis methods (31). Several were supplied by Oligos Etc. or were a gift from Dr. L. Beigelman (Ribozymes Pharmaceuticals Inc., Boulder, CO).

**Binding and Reactivity Measurements.** All reactions were single turnover, with ribozyme (E)<sup>1</sup> in excess of [ $^{32}\text{P}$ ]-5'-end-labeled oligonucleotide substrate (S) and were carried out at 50 °C in 50 mM NaMES buffer, pH 6.6, and 10 mM MgCl<sub>2</sub> (32). Reactivity from the E·S complex was obtained by measuring  $(k_{\text{cat}}/K_{\text{m}})^{\text{G,obs}}$ , the second-order rate constant for the reaction, E·S + G<sub>OH</sub> → products. At least three concentrations of G were used to obtain the dependence of the observed rate constant on [G], the slope of which gives  $(k_{\text{cat}}/K_{\text{m}})^{\text{G,obs}}$ . There is evidence that  $(k_{\text{cat}}/K_{\text{m}})^{\text{G,obs}}$  is limited by the chemical step under the reaction conditions used here (33).

The equilibrium dissociation constant for the E·S complex,  $K_{\text{d}}^{\text{E}}$ , was obtained by following reactions with varying [E]. These experiments were carried out under conditions such that the  $K_{1/2}$  obtained from each concentration dependence reflects a true equilibrium dissociation constant (34, 35). We were interested in obtaining the effects of functional group substitutions on tertiary binding. However, removal of functional groups contributing tertiary binding also results in small effects (<4-fold) on the stability of the P1 duplex. These were corrected to obtain values of  $K_{\text{tert}}^{\text{obs}}$  as follows.

<sup>1</sup> Abbreviations: E refers generically to the *Tetrahymena* ribozyme and S to the oligonucleotide substrate, <sub>5</sub>CCCUCUAAAAA. The individual ribozymes and substrates are defined in Scheme 1. IGS refers to the internal guide sequence of the ribozyme, <sub>5</sub>GGAGGG (see Figure 2). NaMES is sodium 2-(*N*-morpholino)ethanesulfonic acid.

Previous work has provided strong evidence that the P1 duplex of the ribozyme has the same stability as a model P1 duplex in solution (36, 37). Hence, the energetic effects of functional group substitutions on P1 duplex stability were obtained by measuring the equilibrium dissociation constant for a model P1 duplex,  $K_d^{\text{IGS}}$ , with and without each functional group substitution. The value of  $K_d^{\text{IGS}}$  was measured for binding to an oligonucleotide strand having the same sequence as the internal guide sequence of the ribozyme (IGS'  $\equiv$  5'GGAGGGA) using substrate inhibition, as described previously (15). The ratio  $K_d^{\text{IGS}}/K_d^{\text{E}}$  gives the observed tertiary stabilization,  $K_{\text{tert}}^{\text{obs}}$ , which represents the ground state stabilization in addition to that obtained from base pairing; in the absence of tertiary stabilization,  $K_{\text{tert}}^{\text{obs}}$  is equal to 1.

**Calculation of  $\Delta\Delta G^\ddagger$ ,  $\Delta\Delta G_{\text{bind}}$ , and Available Binding Energy.** Values for  $\Delta\Delta G^\ddagger$  and  $\Delta\Delta G_{\text{bind}}$  were obtained from eqs 1 and 2;  $R = 0.00198 \text{ kcal mol}^{-1} \text{ K}^{-1}$  and  $T = 323 \text{ K}$  (50 °C).

$$\Delta\Delta G^\ddagger = -RT \ln[(k_{\text{cat}}/K_m)^{\text{G,rel}}] \quad (1)$$

$$\Delta\Delta G_{\text{bind}} = -RT \ln(K_{\text{tert}}^{\text{obs,rel}}) \quad (2)$$

The relative values used to obtain  $\Delta\Delta G^\ddagger$  and  $\Delta\Delta G_{\text{bind}}$  in Figure 1 are defined as

$$(k_{\text{cat}}/K_m)^{\text{G,rel}} = (k_{\text{cat}}/K_m)^{\text{G,obs}(-3\text{d,rS})} / (k_{\text{cat}}/K_m)^{\text{G,obs}(rS)}$$

$$K_{\text{tert}}^{\text{obs,rel}} = K_{\text{tert}}^{\text{obs}(-3\text{d,rS})} / K_{\text{tert}}^{\text{obs}(rS)}$$

The relative values in Figure 4 for individual functional groups are defined as

$$(k_{\text{cat}}/K_m)^{\text{G,rel}} = (k_{\text{cat}}/K_m)^{\text{G,obs}}[\text{without group present}] / (k_{\text{cat}}/K_m)^{\text{G,obs}}[\text{with group present}]$$

$$K_{\text{tert}}^{\text{obs,rel}} = K_{\text{tert}}^{\text{obs}}[\text{without group present}] / K_{\text{tert}}^{\text{obs}}[\text{with group present}]$$

The relative values in Figure 5 are defined as

$$(k_{\text{cat}}/K_m)^{\text{G,rel}} = (k_{\text{cat}}/K_m)^{\text{G,obs}[dI22 \cdot (-3\text{d,rS})]} / (k_{\text{cat}}/K_m)^{\text{G,obs}[E \cdot S]}$$

$$K_{\text{tert}}^{\text{obs,rel}} = K_{\text{tert}}^{\text{obs}[dI22 \cdot (-3\text{d,rS})]} / K_{\text{tert}}^{\text{obs}[E \cdot S]}$$

with E/S denoting each of the ribozyme/substrate pairs investigated (Scheme 1).

In Figure 5, the available binding energy of a given ribozyme/substrate pair is given relative to that of dI22 and -3d,rS, the ribozyme/substrate pair that has the least available binding energy in the set of ribozymes and substrates studied. The tertiary binding energy provided by each functional group was measured previously by removal of single functional groups from the WT ribozyme and unmodified oligonucleotide [Figure 2B (15, 29, 38–41)]. As described previously (15, 29), the value for the 2'-OH group of G22 was obtained by removal of the functional group from the ribozyme/oligonucleotide complex with rP (5'-CCCUCU); because rP has greater tertiary stabilization than rS, the full energetic effect from removal of this functional

group is expressed in binding. Controls showed that the removal of several other functional groups with bound rS or rP gave the same energetic effect (refs 15, 29, and 38–41 and results herein). The values for the binding energy of individual functional groups have been summed to calculate the available binding energy of each ribozyme/substrate pair used in the analysis of Figure 5 (X-axis). This assumption of energetic additivity is supported by the fit to the two-state model (Figure 5, solid lines).

**Model for the Two-State Binding of Substrate.** The lines in Figure 5 below show the binding energy and reactivity predicted for each ribozyme/substrate pair from the threshold model for substrate binding (15). According to this model, there are only two states in which the substrate can bind, the open complex or the closed complex (Figure 2A). There is evidence that the closed complex is required for reaction (35, 40, 42, 43, 45) so that only the fraction of substrate bound in the closed complex ( $F_c$ ) is assumed to be reactive, reacting with G with a second-order rate constant of  $(k_{\text{cat}}/K_m)^{\text{G}}$  from the closed complex. The predicted reactivity,  $(k_{\text{cat}}/K_m)^{\text{G,pred}}$ , is then obtained from eq 3a.

$$(k_{\text{cat}}/K_m)^{\text{G,pred}} = F_c [(k_{\text{cat}}/K_m)^{\text{G}}] \quad (3a)$$

$$F_c = K_{\text{dock}} / (1 + K_{\text{dock}}) \quad (3b)$$

$$(k_{\text{cat}}/K_m)^{\text{G,pred}} = [K_{\text{dock}} / (1 + K_{\text{dock}})] [(k_{\text{cat}}/K_m)^{\text{G}}] \quad (3c)$$

The rate of reaction from the closed complex,  $(k_{\text{cat}}/K_m)^{\text{G}}$ , is assumed to be unaffected by each functional group substitution so that the value for reaction of rS with the WT ribozyme is used throughout [ $(k_{\text{cat}}/K_m)^{\text{G,obs}} = 6.4 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$ ]. This is supported by the observation that  $(k_{\text{cat}}/K_m)^{\text{G,obs}}$  for reaction of -3d,rS with the WT ribozyme and for reaction of rS with I22 is the same, within error, as that of rS with the WT ribozyme (see Results and Discussion and ref 40) and by previous observations that reaction of E·S·G is the same for WT/rS, dG22/rS, and dG25/rS enzyme/substrate pairs [(29); note that bound G stabilizes the closed complex (34), so that rS binds to dG22 and dG25 predominantly in the closed complex in the presence of bound G].

The fraction of substrate bound in the closed complex ( $F_c$ ) is determined by the equilibrium constant for docking,  $K_{\text{dock}}$  (Figure 2A), according to eq 3b.

Thus,  $(k_{\text{cat}}/K_m)^{\text{G,pred}}$  is related to  $(k_{\text{cat}}/K_m)^{\text{G}}$  and  $K_{\text{dock}}$  according to eq 3c. The value of  $K_{\text{dock}}$  is obtained as follows.  $K_{\text{dock}}$  equals 30 for rS and the WT ribozyme (15). Removal of a functional group that contributes tertiary stabilization decreases the value of  $K_{\text{dock}}$  by an amount corresponding to the tertiary stabilization provided by that functional group. The tertiary stabilization provided by each P1 functional group is known ( $\Delta\Delta G_{\text{dock}}$ ), as summarized in Figure 2B, and is related to the change in  $K_{\text{dock}}$  as follows:  $\Delta\Delta G_{\text{dock}} = -RT \ln K_{\text{dock}}^{\text{rel}}$ ;  $K_{\text{dock}}^{\text{rel}} = K_{\text{dock}}(\text{WT/rS}) / K_{\text{dock}}(\text{E/S})$ . To obtain  $K_{\text{dock}}$  for ribozyme/substrate pairs that have more than one functional group substitution, the individual tertiary binding energies were assumed to be additive. (This assumption is supported by the data shown in Figure 5.)

The predicted ground state stabilization beyond that obtained from base pairing,  $K_{\text{tert}}^{\text{pred}}$ , is given by eq 4, which is derived from the expression  $K_d^{\text{E}} = K_d^{\text{IGS}} / (1 + K_{\text{dock}})$  in Figure 2A.

$$K_{\text{tert}}^{\text{pred}} = K_{\text{d}}^{\text{IGS}}/K_{\text{d}}^{\text{E}} = 1 + K_{\text{dock}} \quad (4)$$

In Figure 5, the predicted binding and reactivity of a given ribozyme/substrate pair are plotted relative to the values for the dI22 ribozyme/−3d,rS pair. The  $\Delta\Delta G^{\ddagger,\text{pred}}$  and  $\Delta\Delta G_{\text{bind}}^{\text{pred}}$  values that determine the lines in Figure 5 are obtained from eqs 5a and 5b.

$$\Delta\Delta G^{\ddagger,\text{pred}} = -RT \ln[(k_{\text{cat}}/K_{\text{m}})^{\text{G,pred(rel)}}] \quad (5a)$$

$$\Delta\Delta G_{\text{bind}}^{\text{pred}} = -RT \ln[K_{\text{tert}}^{\text{pred(rel)}}] \quad (5b)$$

The values of  $(k_{\text{cat}}/K_{\text{m}})^{\text{G,pred(rel)}}$  and  $K_{\text{tert}}^{\text{pred(rel)}}$  were obtained by dividing the values of  $(k_{\text{cat}}/K_{\text{m}})^{\text{G,pred}}$  (eq 3c) and  $K_{\text{tert}}^{\text{pred}}$  (eq 4) for the dI22 ribozyme/−3d,rS pair by the respective  $(k_{\text{cat}}/K_{\text{m}})^{\text{G,pred}}$  and  $K_{\text{tert}}^{\text{pred}}$  values for each ribozyme/substrate pair.

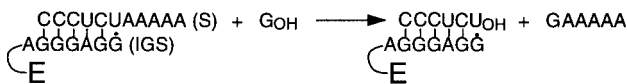
**Estimation of Errors.** There were variations of rate constants of up to 3-fold between independent experiments. The variation in the ratios of rate constants was less,  $\pm 20\%$ , within the same experiment. Values of  $K_{\text{d}}^{\text{IGS}}$  and  $K_{\text{d}}^{\text{E}}$  obtained from the rate measurements varied by  $\pm 30\%$  between independent experiments.

## RESULTS AND DISCUSSION

The *Tetrahymena* group I ribozyme (E) cleaves an oligonucleotide substrate (S) using a guanosine nucleophile ( $\text{G}_{\text{OH}}$ ) with a rate enhancement of  $10^{11}$ -fold (Scheme 1). Considerable previous work has provided strong evidence for two-step binding of the ribozyme's oligonucleotide substrate (Figure 2A) (36, 37, 42–45). In the first step, the substrate base pairs with the ribozyme's internal guide sequence (IGS) forming the P1 duplex to give an open complex. In the second step, the P1 duplex docks into the catalytic core via tertiary interactions to give a closed complex (Figure 2B) (15, 29, 35, 38–41, 46–48).

Initially, the energetic effect of a specific 2'-OH group on the oligonucleotide substrate was investigated by replacing

Scheme 1<sup>a</sup>



Abbreviation	Substrate
rS	rC rC rC rU rC rU rA rA rA rA
-2d,rS	rC rC rC rU dC rU rA rA rA rA
-3d,rS	rC rC rC dU rC rU rA rA rA rA

Abbreviation	Ribozyme's Internal Guide Sequence
WT	22 rG rG rA 25 rG rG 27 rG ——— E
dG22	dG rG rA rG rG rG ——— E
I22	rI rG rA rG rG rG ——— E
dI22	dI rG rA rG rG rG ——— E
dG25	rG rG rA dG rG rG ——— E

<sup>a</sup> r refers to a ribose residue, d to 2'-deoxyribose, and I to inosine.

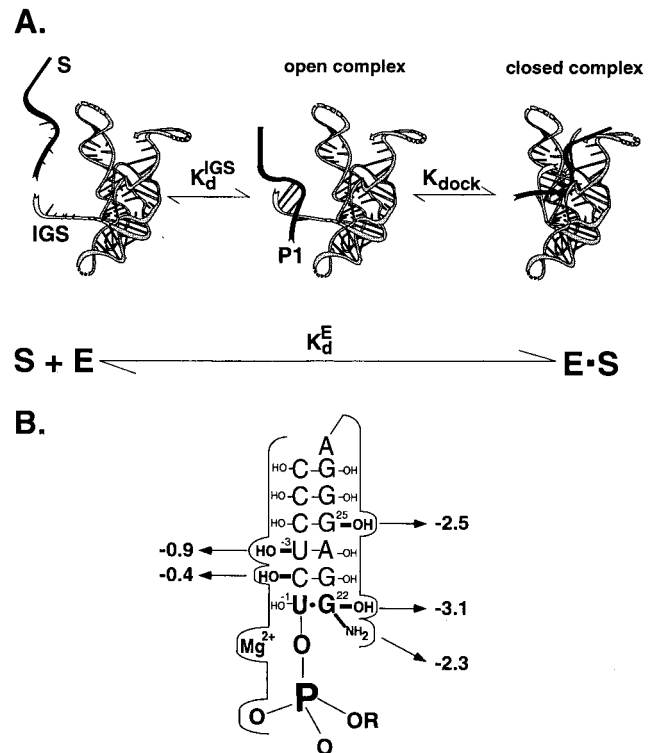


FIGURE 2: Two-step binding of the ribozyme's oligonucleotide substrate. (A) The oligonucleotide substrate (S) first forms an open complex. In the open complex, S is held only by base-pairing interactions with the internal guide sequence (IGS) of the ribozyme (E), forming the P1 duplex. The P1 duplex then docks into tertiary interactions with the ribozyme to form the closed complex (36, 37, 42–44).  $K_{\text{d}}^{\text{E}}$  is the equilibrium constant for dissociation of the E·S complex,  $K_{\text{d}}^{\text{IGS}}$  is the dissociation constant for the open complex, and  $K_{\text{dock}}$  is the equilibrium constant for docking into the tertiary interactions, such that  $K_{\text{d}}^{\text{E}} = K_{\text{d}}^{\text{IGS}}/(1 + K_{\text{dock}})$ . (B) In the closed complex, the oligonucleotide substrate is held by both base-pairing interactions with the internal guide sequence of the ribozyme (IGS  $\equiv$  5'-GGAGGG) and tertiary interactions. The 2'-OH groups and the exocyclic amino group of the G·U wobble pair shown in bold provide tertiary stabilization to the P1 duplex. The tertiary stabilization provided by the individual groups discussed herein is shown in kcal/mol (50 °C) (15, 29, 35, 38–41, 46). The ribozyme active site is represented schematically by the thin outline.

it with a 2'-H (Scheme 1, rS  $\rightarrow$  −3d,rS). This 2'-OH has been proposed to make a tertiary interaction in the ribozyme active site (Figure 2B) (38–40, 47, 48). Replacement by the 2'-H destabilizes binding by 0.9 kcal/mol but does not affect reaction of the bound substrate (E·S), consistent with previous results (Figure 1A) (15, 38–40, 47, 48). Surprisingly, with a modified ribozyme that lacks two of the tertiary interactions made by the P1 duplex (Scheme 1, dI22), the 2'-H substitution does not destabilize binding. Instead, this substitution specifically destabilizes the transition state by 1.0 kcal/mol without affecting binding (Figure 1B; see Materials and Methods). The catalytic effect of the 2'-OH group was also surprising because this group is distant from the site of the chemical reaction and is held rigidly within the P1 duplex, rendering it unlikely to interact directly with the groups undergoing charge rearrangements during the reaction.

The following analysis provides a simple explanation for the above paradox. The modified ribozyme, dI22, lacks two of the functional groups on the P1 duplex that make tertiary interactions with the ribozyme core, the exocyclic amino

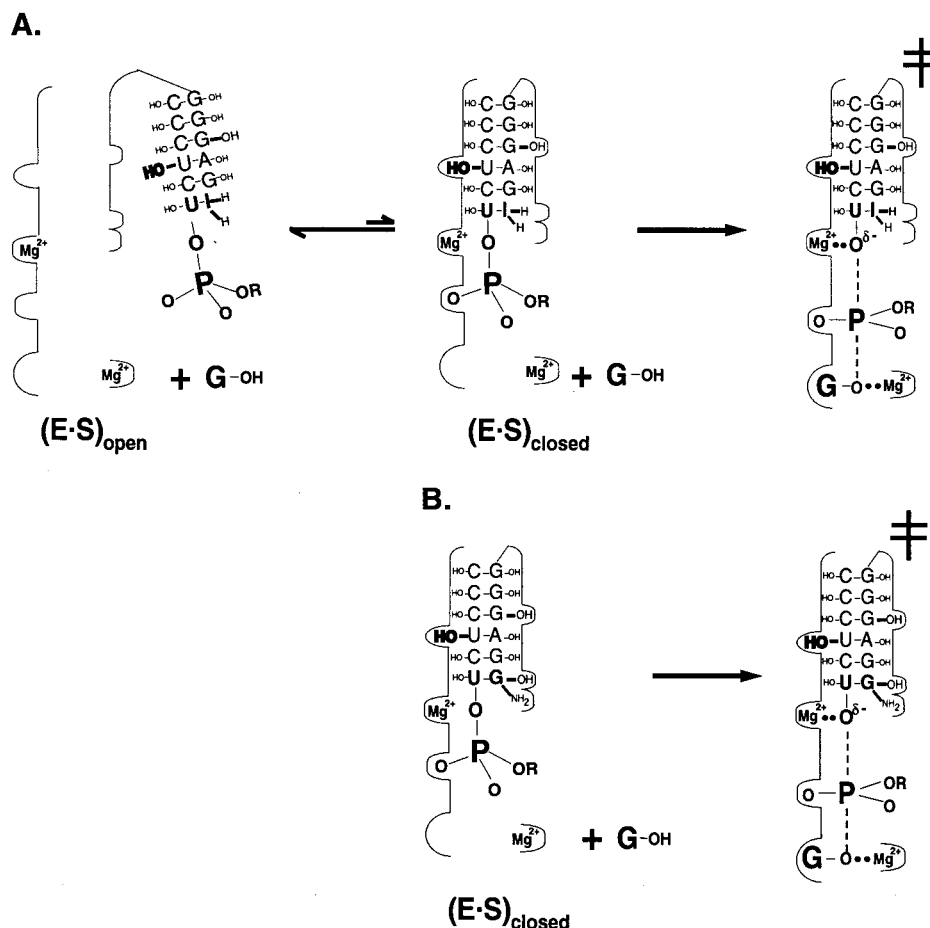


FIGURE 3: Uniform binding or specific transition state stabilization by the same functional group in different ribozyme contexts. Reaction of rS with dI22 (A) and with WT (B). The 2'-OH at position -3 that is replaced with a 2'-H is shown in bold. Transition state (‡) interactions between a  $\text{Mg}^{2+}$  ion and the partially negatively charged phosphoryl bridge oxygen (49) and between a  $\text{Mg}^{2+}$  ion and the 3' oxygen of G (50) are shown explicitly. With the dI22 ribozyme (A), rS is bound predominantly in the open complex (see Figure 2A) and hence is held solely by base-pairing interactions. Docking into the closed complex is required prior to chemical cleavage. With the WT ribozyme (B), rS is repositioned in the closed complex and binding is stabilized by tertiary interactions in addition to base-pairing interactions.

group and the 2'-OH group of G22 (Figures 1B and 2B). The substrate containing the specific 2'-OH group, rS, binds to the dI22 ribozyme with the same affinity as it binds to an oligonucleotide with the sequence of the ribozyme's IGS ( $K_d = 1.5$  and  $1.6 \mu\text{M}$ , respectively; see Materials and Methods), providing strong support for binding in the open complex (Figure 3A; refs 15, 36, 37). Thus, the binding energy from the available tertiary interactions is insufficient to dock the substrate into the active site in the ground state, and the substrate is localized to the ribozyme simply via base-pairing interactions [Figure 3A, (E·S)<sub>open</sub>]. Removal of the specific 2'-OH group of the substrate does not decrease binding because the 2'-OH group does not make a tertiary interaction with the ribozyme in the ground state. However, to react, the substrate has to dock into the closed complex in which it is positioned with respect to the catalytic Mg ions and with respect to the guanosine nucleophile [Figure 3A, (E·S)<sub>closed</sub>] (49, 50). Thus, removal of the 2'-OH group, which makes a tertiary interaction with the ribozyme at a distance from the site of chemical transformation (38–40, 47, 48), specifically destabilizes the transition state and slows reaction of bound substrate.

In contrast to its behavior with the dI22 ribozyme, the rS substrate binds more strongly to the WT ribozyme than it binds to an oligonucleotide with the sequence of the

ribozyme's IGS ( $K_d = 8$  and  $250 \text{ nM}$ , respectively; see Materials and Methods). Thus, the binding energy from the available tertiary interactions is sufficient to dock the substrate within the active site in the ground state [Figure 3B, (E·S)<sub>closed</sub>]. As a result, the 2'-OH makes a tertiary interaction with the ribozyme in the ground state as well as in the transition state. Removal of the specific 2'-OH group from the substrate therefore destabilizes both the ground state and the transition state, giving a uniform binding effect. Thus, binding is affected but the rate of the chemical step is not (Figure 3B).

Previous descriptions of enzymatic functional groups have categorized specific functional groups as having distinct catalytic signatures, providing uniform binding, specific transition state stabilization, or a hybrid of the two (e.g., refs 26 and 40). However, the results above demonstrate that a functional group contributing uniform binding with the WT ribozyme instead contributes specific transition state stabilization with a mutant ribozyme. This indicates that the catalytic signature of a group can change depending on context and extends the analysis of Albery and Knowles (26).

The model used to account for these results predicts that each of the functional groups involved in tertiary binding should be capable of contributing either uniform binding or specific transition state stabilization, analogous to the effects

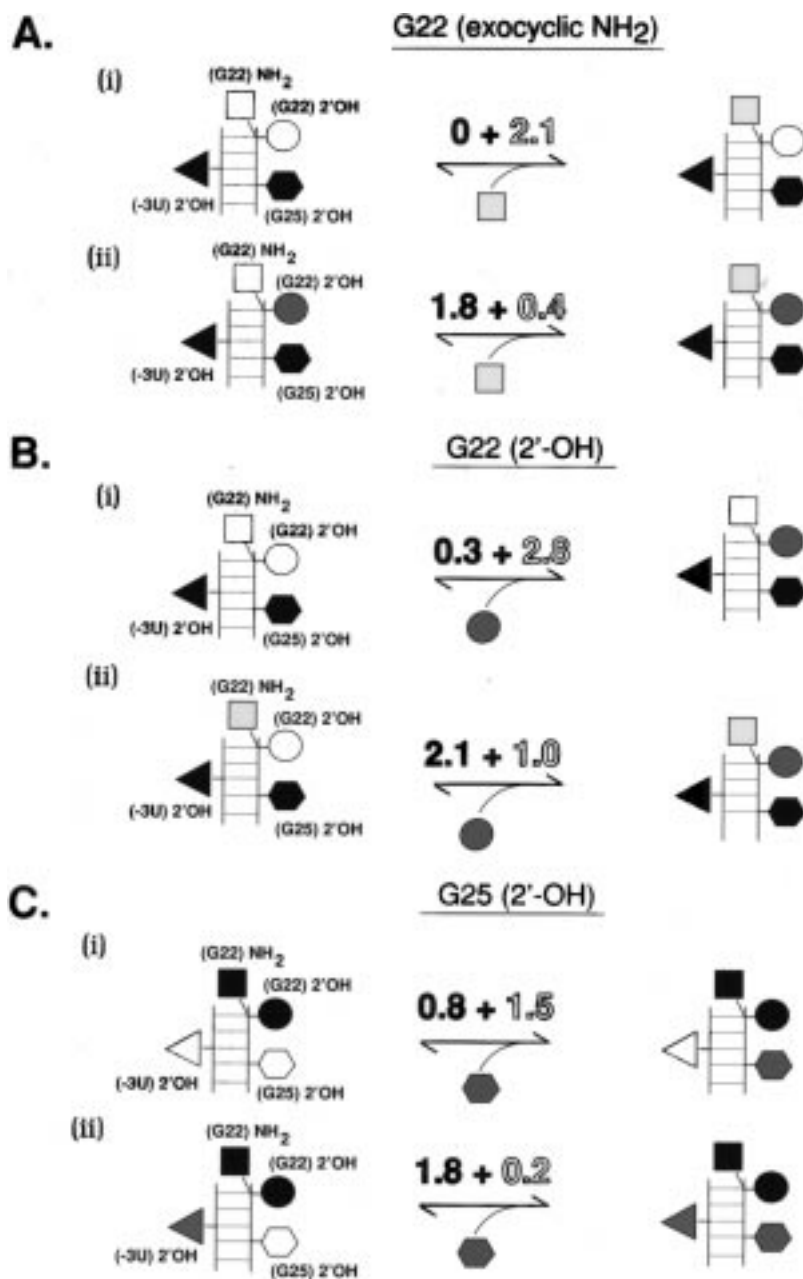


FIGURE 4: Context-dependent contributions of P1 functional groups. Results are shown for the exocyclic amino group of G22 (A), the 2'-hydroxyl of G22 (B), and the 2'-hydroxyl of G25 (C). In each comparison, the unfilled symbols indicate the absence of a particular functional group. A particular functional group that is added is represented by a colored symbol. The black symbols represent groups that are present but not modified in each comparison. Each number represents the favorable energetic effect of adding in a particular functional group in kcal/mol (50 °C). Filled numbers represent effects on binding ( $\Delta\Delta G_{\text{bind}}$ ), and unfilled numbers represent effects on reactivity ( $\Delta\Delta G^\ddagger$ ). In each comparison, the total amount of stabilization provided by a functional group is, within error, independent of the context.

of the 2'-OH group of S described above. Further, whether a functional group contributes uniform binding or specific transition state stabilization is solely determined by the total amount of binding energy available from the functional groups present on the P1 duplex. To test these predictions, we analyzed the energetic effects of three other P1 functional groups in the context of modified ribozymes and substrates that have different amounts of available binding energy (Figure 4).

In the absence of the 2'-OH group at G22 [Figure 4A (i), the missing 2'-OH is represented by the open circle], the exocyclic amino group at the cleavage site, represented by the yellow square, contributes 2.1 kcal/mol toward stabilizing the transition state without affecting ground state binding.

In contrast, when the 2'-OH group is present at G22 [Figure 4A (ii), blue circle], the exocyclic amino group contributes 1.8 kcal/mol to ground state binding, whereas the additional transition state stabilization component is only 0.4 kcal/mol. Analogously, in the comparisons of panels B and C of Figure 4, a given functional group provides a fixed total energetic contribution, but the contribution to binding versus reactivity of the bound substrate varies with the context. Each group predominantly displays uniform binding in the context of the WT ribozyme [(ii) in each panel of Figure 4] but provides specific transition state stabilization in a context in which the available binding energy is insufficient to position the substrate within the active site [(i) in each panel of Figure 4]. Thus, the amount of a functional group's binding energy

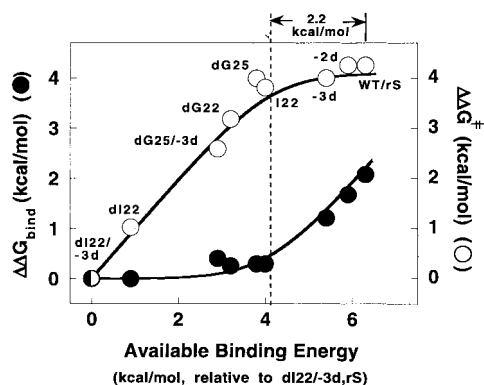


FIGURE 5: The total available binding energy determines whether added functional groups enhance binding or the chemical step. The modifications in E and S (Scheme 1) are shown explicitly.  $\Delta\Delta G_{\text{bind}}$  and  $\Delta\Delta G^{\ddagger}$  were obtained as described in Materials and Methods. The available binding energy for each E/S pair (see Materials and Methods) is depicted relative to that for the dI22 ribozyme with  $-3d,rS$ . The lines are fit to  $\Delta\Delta G_{\text{bind}}^{\text{pred}}$  and  $\Delta\Delta G^{\ddagger,\text{pred}}$ , the binding and reactivity values predicted from a two-state “threshold” model for substrate binding (see text and Materials and Methods). Loss of greater than 2.2 kcal/mol of binding energy relative to the binding energy with rS and the WT ribozyme results in binding predominantly in the open complex (15). This sets an energetic threshold, represented by the dashed line, that separates ribozyme/substrate complexes that are predominantly in the open complex from those that are predominantly in the closed complex.

that is expressed in the ground state versus the transition state is determined by the amount of total binding energy available from the other tertiary interactions (see also Figure 5 below).

This context-dependent expression of binding energy is quantitatively accounted for by the two-state model for substrate binding shown in Figure 2A. This model is supported by previous physical characterizations of the open and closed complexes, which have shown the two complexes to be structurally distinct, and by considerable previous data on the energetic effects of P1 functional groups, which can be fully explained in terms of two states (15, 29, 35–37, 41–46, 51, 52). According to this model, the substrate can bind only in the open complex or the closed complex, with the binding mode determined solely by the amount of binding energy available. If the available binding energy is greater than a “threshold” amount, then the bound substrate is predominantly in the closed complex, so it reacts with the maximal rate constant. If the available binding energy is insufficient to cross the energetic threshold, then the substrate is bound predominantly in the open complex. In such a case, the bound substrate must dock before the chemical cleavage can occur, so the rate constant for reaction of the bound substrate is reduced.

The binding and reactivity for ribozyme/substrate pairs with varying amounts of total binding energy available from tertiary interactions are plotted in Figure 5. The lines in this figure represent the binding and reactivity predicted by the threshold model (see Model for the Two-State Binding of Substrate in Materials and Methods). The observed reactivities and binding energies fit well to the behavior predicted from the threshold model. Initial increases in binding energy accelerate reaction of the bound substrate by increasing the fraction of the bound substrate that is present in the closed complex. Once the amount of binding energy is sufficient to cross the energetic threshold, represented by the dotted

line (15), the substrate is predominantly docked; subsequent increases in binding energy increase binding without accelerating reaction of the bound substrate. Although the amount of the added binding energy that is expressed in substrate binding versus substrate reactivity varies in the different modified contexts, the sum of the effects for a given functional group on binding and on reactivity is the same, regardless of the context [Figure 4, total energetic contribution in (i) vs (ii) for each comparison]. Thus, the total energetic contribution of each added functional group is independent of the identity of the remaining functional groups. This suggests that the physical interactions made by a group are the same in the WT and mutant contexts, supporting the simple interpretations presented above.

As introduced earlier, it has been widely held that the fundamental difference between simple chemical catalysts and enzymes is the ability of enzymes to use binding interactions away from the site of bond cleavage for catalysis. This use of binding interactions for enzymatic catalysis was strongly suggested by the identification of enzymatic and substrate functional groups in several systems that are at a distance from the site of the chemical transformation yet express their effects on  $k_{\text{cat}}$  (e.g., refs 4–13, 25, and 53–55).

The results here directly demonstrate that binding interactions contribute to enzymatic catalysis; the same group that contributes solely to binding with the wild-type ribozyme can accelerate reaction of bound substrate with modified forms of this RNA enzyme. Even functional groups that are distant from the site of the chemical transformation, such as the 2'-hydroxyl groups investigated herein (Figure 4), can exhibit this behavior.

Binding interactions can be catalytic because they can position the substrate with respect to other substrates and groups that are more commonly referred to as “catalytic”, such as the Mg ions in the *Tetrahymena* ribozyme active site (Figure 3) (49, 50), thereby allowing these groups to exert their maximal effect. Conversely, if a conventional catalytic group were absent, then the catalytic effect of functional groups that position the substrate within the active site would be diminished. This provides an example of how the catalytic contributions of binding interactions and the groups typically referred to as catalytic can be inextricably linked (for related discussions, see refs 4, 11, and 18).<sup>2</sup>

The results here, which directly demonstrate the catalytic role of binding interactions, together with the extensive previous work cited above, provide strong support for the view that binding interactions are fundamental to the enormous rate enhancements achieved by enzymes, both protein and RNA (4, 17). For protein enzymes, the catalytic

<sup>2</sup> Not all binding interactions are expected to exhibit such an inextricable link with catalytic interactions. Some interactions might best be viewed as “tethering”. Binding interactions with substrate or cofactor groups that are remote from the site of chemical transformation and that are not rigidly connected to the site of chemical transformation may serve only to localize the substrate to the enzyme. Such binding interactions would enhance the reaction of free E with free S; i.e., the rate enhancement would be manifested in  $k_{\text{cat}}/K_m$ . However, when the substrate is bound to the enzyme, addition of tethering interactions would not increase the reaction rate ( $k_{\text{cat}}$ ). This is in contrast to the class of binding interactions described here that position the substrate with respect to the catalytic groups, thereby accelerating reaction of bound substrate.

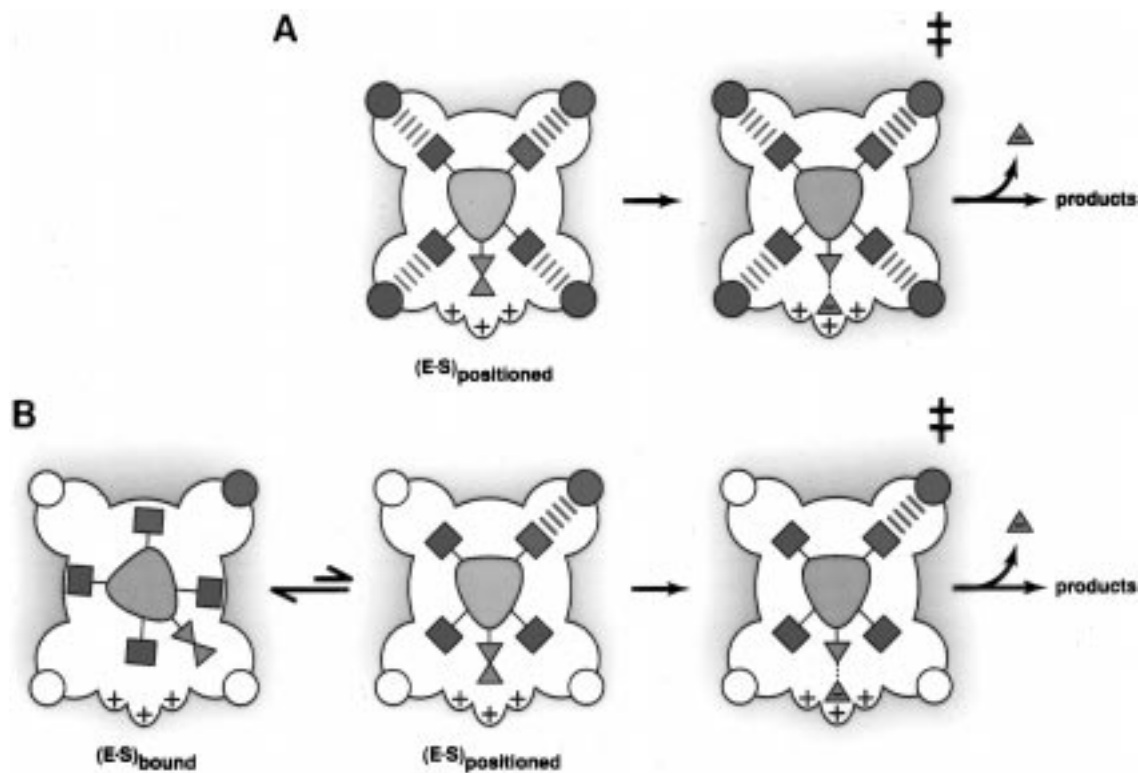


FIGURE 6: Schematic view of the catalytic role of binding interactions in protein enzymes. Reaction of a substrate bound to a wild-type enzyme (A) or bound to a mutated enzyme (B). The enzyme's active site is represented by the outline and by the colored circles, which represent protein residues that interact with functional groups of the substrate. The substrate is represented by the gray shape, and its functional groups are represented by the colored squares. Protein residues deleted via mutagenesis are represented by the open circles in (B). In the transition state ( $\ddagger$ ), the bond between the two functional groups represented by the blue triangles is partially cleaved, generating a buildup of negative charge (denoted by "-"). The "+" symbols represent catalytic groups such as metal ions, hydrogen bond donors, or a general acid that can stabilize this developing negative charge. In (B), the state  $(E\cdot S)_{\text{bound}}$  is meant to depict the increased flexibility and potential alternative binding modes of substrate bound to the mutant enzyme.

role of binding interactions could, in principle, be directly demonstrated using the same approach as that used for the *Tetrahymena* ribozyme in which multiple functional group substitutions were used to reveal the catalytic contributions of binding interactions. This is illustrated schematically with a hypothetical protein enzyme in Figure 6.

In Figure 6A, the green protein residues interact with the green functional groups of the substrate and position the substrate within the active site with respect to the catalytic groups ("+"). These interactions allow the red protein residue to interact with the substrate in the ground state as well as the transition state. Hence, mutation of the red protein residue weakens binding. However, since the green residues are still present and are sufficient to position the bound substrate, mutation of the red residue does not affect reaction of the bound substrate. As a result, mutation of the red residue gives a uniform binding effect. Nevertheless, the catalytic role of the red protein residue in the chemical step would be revealed by mutation of the red residue after mutation of the green residues (Figure 6B). In the absence of the green residues, the available binding energy is insufficient to precisely position the bound substrate in the active site. Because it is not well positioned, the red residue, though present, does not make a stable ground state interaction [ $(E\cdot S)_{\text{bound}}$ ]. Nevertheless, transient formation of the red interaction better positions the bound substrate for reaction [ $(E\cdot S)_{\text{positioned}}$ ]. Thus, mutation of the red residue decreases the rate of reaction by decreasing the fraction of the bound substrate that is well positioned for reaction, thereby

demonstrating the catalytic role of this residue.

The analysis of Figure 6 suggests that assigning a uniform binding role to an enzymatic residue on the basis of the results of single mutations can be misleading. As observed here with the *Tetrahymena* ribozyme, although a single mutation may give a uniform binding effect, a catalytic role of the residue in the chemical step can be masked if the remaining residues provide sufficient binding energy to position the substrate within the active site.

Despite the apparent underlying commonality, a direct demonstration of the catalytic role of binding interactions, analogous to that described for the *Tetrahymena* RNA enzyme, may be difficult, in practice, with protein enzymes. Energetic and structural analyses have shown that mutations in protein active sites can lead to rearrangements (e.g., refs 56–62). The resulting changes in the detailed interactions made by specific functional groups would complicate comparisons of the energetic contributions of specific functional groups in different mutant contexts.

In contrast, RNA enzymes may be particularly amenable to energetic dissections. This is suggested by the results here that the total amount of stabilization provided by each functional group on the P1 duplex is the same in wild-type and modified versions of the *Tetrahymena* ribozyme (Figure 4). The results provide strong evidence that the interactions made by the individual P1 functional groups remain the same upon mutagenesis of the ribozyme. The following features of RNA may in general limit rearrangement in response to site-specific modifications. (i) An RNA active site is likely



to be less close packed and have more solvent-mediated interactions than an active site in a protein due to the limited shapes, sizes, and polarities of RNA side chains. Hence, functional group substitutions may cause smaller disruptions in local structure, resulting in smaller rearrangements to accommodate the disruptions. Further, the preponderance of hydrophobic interactions in proteins relative to RNA might allow for more facile rearrangement upon mutagenesis. (ii) Individual functional group substitutions are readily made in RNA, owing to the power of solid-phase synthetic approaches. Such chemical "mutagenesis" further limits the extent of rearrangements. Mutagenesis with unnatural amino acids can allow for analogous conservative changes in proteins (63–68). (iii) The rigidity of RNA duplexes can limit rearrangement of remaining functional groups upon multiple modifications.

Analysis of the energetic effects of functional group substitutions is further facilitated for the *Tetrahymena* RNA enzyme because of the two-state nature of substrate binding. In this system, a state in which the substrate is bound, but not positioned, can be thermodynamically and structurally distinguished from a state in which the substrate is positioned with respect to the catalytic groups (15, 29, 36–45). This has allowed the use of binding energy to be directly correlated with positioning in the active site (Figure 5). Such an analysis would be difficult if multiple alternative binding modes were possible.

In summary, it has been possible, using the *Tetrahymena* RNA enzyme, to demonstrate that a functional group that contributes to binding in one context can contribute directly to catalysis in another context, underscoring the inextricable link between binding energy and enzymatic catalysis. The results also suggest that the role of active site interactions can change during the evolution of an enzyme. For some primitive enzymes, a functional group may have specifically stabilized the transition state because the other binding interactions were not sufficient to precisely position the substrates with respect to the catalytic groups in the ground state, analogous to modified *Tetrahymena* ribozymes and the hypothetical enzyme of Figure 6B. Upon evolution of additional interacting groups to position the substrates, this same functional group could then display uniform binding upon mutagenesis, as shown in Figure 6A.

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