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MECHANISTIC ASPECTS OF ENZYMATIC CATALYSIS: Lessons from Comparison of RNA and Protein Enzymes[1]

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ABSTRACT

A classic approach in biology, both organismal and cellular, is to compare morphologies in order to glean structural and functional commonalities. The comparative approach has also proven valuable on a molecular level. For example, phylogenetic comparisons of RNA sequences have led to determination of conserved secondary and even tertiary structures, and comparisons of protein structures have led to classifications of families of protein folds. Here we take this approach in a mechanistic direction, comparing protein and RNA enzymes.

The aim of comparing RNA and protein enzymes is to learn about fundamental physical and chemical principles of biological catalysis. The more recently discovered RNA enzymes, or ribozymes, provide a distinct perspective on long-standing questions of biological catalysis. The differences described in this review have taught us about the aspects of RNA and proteins that are distinct, whereas the common features have helped us to understand the aspects that are fundamental to biological catalysis. This has allowed the framework that was put forth by Jencks for protein catalysts over 20 years ago (1) to be extended to RNA enzymes, generalized, and strengthened.

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[1]This review is dedicated to WP Jencks on the occasion of his seventieth birthday in honor of his contributions to the understanding of enzymes.

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INITIAL MECHANISTIC QUESTIONS RAISED FROM THE DISCOVERY OF RNA ENZYMES

In 1982, it was shown that an intron within pre-rRNA from *Tetrahymena thermophila* could excise itself, or self-splice, in the absence of proteins (2). This discovery generated excitement on several fronts. The ability of RNA to speed reactions provided a possible answer to the evolutionary chicken-and-egg problem of whether macromolecules that provide information storage came before those that provide catalytic function or vice versa, as RNA could fulfill both roles (3–5). The ability of an RNA molecule to cleave other RNA molecules in a sequence-dependent fashion suggested a potential therapeutic approach for blocking the expression of deleterious proteins at the RNA level (6, 7, 7a).

From a mechanistic perspective, the ability of RNA to act as an enzyme was not generally expected. The surprise upon discovering RNA to be catalytic can be appreciated from the simple observation that RNA lacks the functional groups analogous to those on protein side chains that are most frequently identified as catalytic residues: the imidazole of His, the carboxylate of Asp and Glu, the alkyl amine of Lys, and the sulfhydryl of Cys (Figure 1). The low diversity of RNA side chains, the high charge and flexibility of its backbone, and the resultant limitations on precise positioning were also expected to limit the catalytic capabilities of RNA.

The ability of RNA to self-splice despite these apparent limitations raised several immediate questions that are outlined below. Initial answers to these questions are presented in this section to provide background about RNA catalysis.

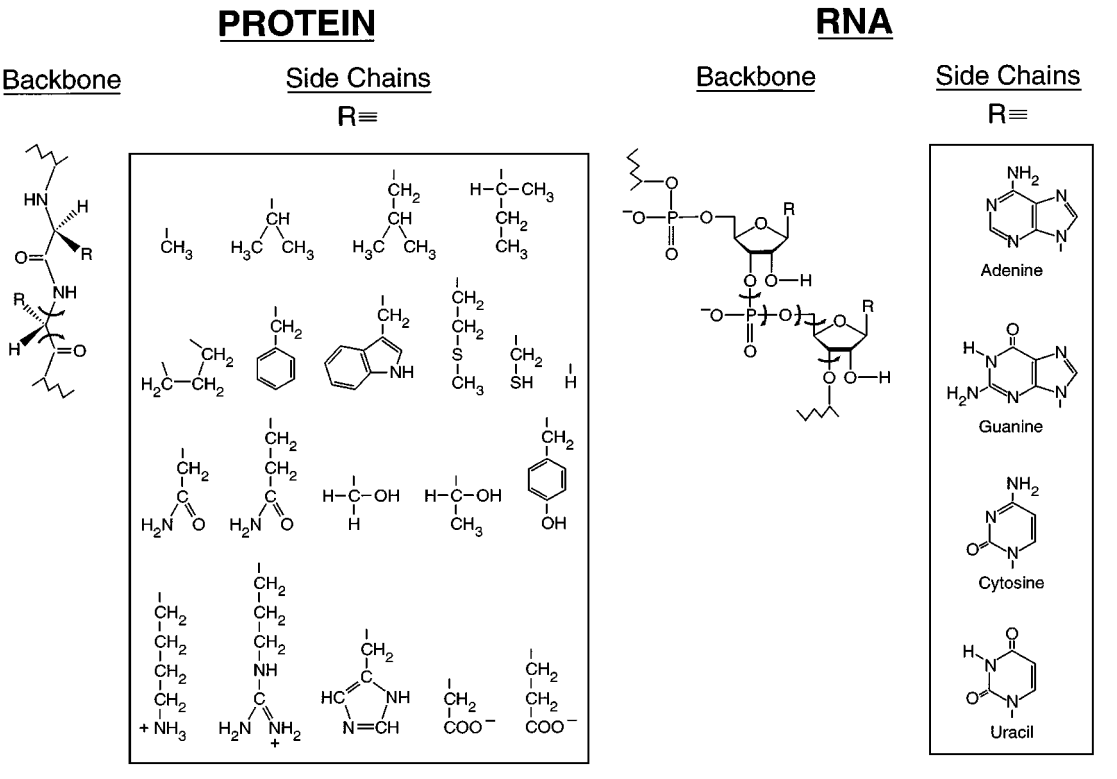


Figure 1 Comparison of the backbone and side chains of protein and RNA. Each of the two and five freely rotatable bonds in the protein and RNA backbones, respectively, is indicated by a curved arrow.

The comparisons between RNA and protein enzymes presented in the subsequent sections develop these and other mechanistic questions in greater depth and suggest some possible answers. Because there have been few treatments of mechanistic aspects of RNA catalysis, we present in more detail the specific features of the RNA catalysts. Several reviews provide general background as well as discussions of other implications of RNA catalysis (8–11). Background information can also be found in several treatments of the mechanistic enzymology of proteins (1, 12–14).

Can RNA Act As a True Catalyst?

The first reaction shown to be facilitated by RNA was a self-processing or self-splicing reaction (2), raising the question among mechanistic enzymologists of whether or not RNA could act as a true catalyst, facilitating successive reactions without itself being changed. This concern was put to rest by the discovery that the RNA component of RNase P was responsible for the catalytic processing of tRNA precursors (15). In addition, the self-splicing group I intron from *T. thermophila* could be engineered into a multiple turnover RNA enzyme (16). Indeed, newly discovered ribozymes that facilitate intramolecular reactions are now routinely converted into RNA enzymes that can catalyze multiple turnover reactions (17–25).

An unexpected benefit of this approach has been that reactions of RNA enzymes engineered to utilize exogenous substrates can more readily be dissected into individual reaction steps. There are now kinetic and thermodynamic frameworks for several classes of ribozymes (20, 26–31). These frameworks have allowed interpretation of the effects of mutations and single functional group substitutions at the level of rate and equilibrium constants for individual reaction steps and have been crucial in providing mechanistic insights into ribozyme action (e.g. 20, 25, 32–55).

How Good Is RNA As a Catalyst?

Albery & Knowles presented a framework for evaluating catalytic efficiency, which, in its simplest form, is that an enzyme that operates with maximal efficiency will catalyze the reaction of every substrate molecule that it encounters; the reaction will be limited by physical steps of diffusion rather than by the chemical transformation (56). Several ribozymes meet this criterion for catalytic perfection, cleaving essentially every RNA substrate molecule that they bind (26–28, 30).[2]

[2]Substrate binding for most ribozymes involves duplex formation, which is considerably slower than diffusional encounter, as it requires an initial nucleation event (26). It remains to be determined if a structured ribozyme active site can speed this binding event either via electrostatic steering or by preorienting one of the duplex strands within the active site (e.g. 57–59).

The description of RNA enzymes as "perfect" catalysts is presented solely to demonstrate that RNA can be an effective catalyst [for lively discussion of catalytic perfection see, for example, (60–63)]. The following additional considerations of RNA enzymes from a standpoint of catalytic perfection illustrate some idiosyncracies of ribozymes. A K_m that is greater than the physiological concentration of substrate ensures that the enzyme is not occupied with bound substrate or intermediate and is free to encounter and react with another substrate molecule (1, 14, 60, 64). However, most ribozymes facilitate intramolecular reactions, self-splicing, or self-cleavage, so a physiological concentration of substrate cannot be defined. Tight binding and slow release of intermediates may play a biological role in self-splicing (see "Comparisons of the Ability of RNA and Protein Enzymes to Carry Out Complex Functions"), and this could explain why RNA enzymes engineered to act with multiple turnover are typically easily saturated and have overall turnover limited by slow product release (26, 27). However, increasing turnover rates for ribozymes can be straightforward. When substrate (and product) recognition involves base pairing, turnover can often be improved simply by introducing mismatches or shortening the ribozyme's recognition strand (25, 36, 43); when substrate recognition involves tertiary interactions, there also appear to be many mutations that can improve turnover by weakening these interactions (32).

Another criterion for evaluating catalytic prowess is the rate enhancement relative to the corresponding reaction in solution. The group I ribozyme from *Tetrahymena* and the RNA component of RNase P have rate constants for their chemical steps of $\sim 3\ sec^{-1}$, corresponding to a rate enhancement of $\sim 10^{11}$-fold relative to the estimated rate constant for the solution reaction (26, 28). This increase is within the range of rate enhancements observed with protein enzymes (65) and shows that RNA must be taken seriously as a catalyst. Nevertheless, protein enzymes can catalyze these same reactions to an even greater extent, and the rate enhancement achieved by some other ribozymes is less impressive. Features of these catalysts that could be responsible for these differences are discussed in the following sections.

How Extensive Is the Repertoire of Reactions that RNA Can Catalyze?

All of the known naturally occurring ribozymes catalyze reactions at phosphoryl centers, i.e. transphosphoesterification and phosphate hydrolysis reactions. Experiments designed to remove protein components from ribosomes have suggested that peptidyl transfer in protein synthesis may be catalyzed by RNA components of the ribosome (66). The *Tetrahymena* group I ribozyme provides a small (10-fold) rate advantage for hydrolysis of an ester, a reaction at a carbon center (67). In vitro selections have also begun to widen RNA's catalytic

repertoire, although the rate enhancements are often small (68–70; see also 70a, 71). In the following sections, we compare RNA and proteins in an attempt to understand what features of each broaden and restrict the catalytic range of these macromolecules.

What Structural Features of an RNA Molecule Confer Catalysis?

There exist a multitude of atomic resolution crystal structures of protein enzymes that have, in many cases, provided insight into structural features that contribute to catalysis. The need to obtain high-resolution structures of catalytic RNAs has been apparent for the past 15 years. Nevertheless, there are currently structures for only a small number of complex RNAs and only one structure of a catalytic RNA, the hammerhead (72–85). Furthermore, the three-dimensional (3D) structure of the hammerhead ribozyme apparently does not correspond to the catalytic conformation (83, 84); a structural rearrangement is required to account for the stereochemistry and the involvement of residues implicated by site-directed mutagenesis (86).

Despite the current absence of structural information that is mechanistically revealing, considerable information is available from structure-function studies about interactions involved in catalysis and their energetic contributions, especially for the most extensively studied ribozymes, the hammerhead ribozyme and the group I ribozyme from *Tetrahymena*. In the following sections, we also use the limited but informative RNA structures and the implied structural differences between RNA and proteins to consider potential catalytic distinctions and similarities. The ability to make RNA in large quantities, via in vitro transcription and solid phase synthesis; the development of site-specific labeling of RNA; two-dimensional (2D) and 3D nuclear magnetic resonance (NMR) approaches; and the recent structural successes should stimulate further structural work and lead to a wealth of structural information over the coming years, allowing these ideas to be tested, refined, and extended (79–85, 87–92).

COMPARISONS OF CATALYTIC STRATEGIES OF RNA AND PROTEIN ENZYMES

As described in the above section, RNA appears to lack many characteristics considered important for catalysis by protein enzymes. Below we compare the catalytic strategies of RNA and proteins using differences between these catalysts to bring specific mechanistic questions into focus. The comparisons largely rely on results with the *Tetrahymena* group I ribozyme and the hammerhead ribozyme. The analyses suggest that although RNA is deficient in general acid-base catalysis and certain types of covalent catalysis, it may be particularly

adept at using metal ions. Indeed, all known, naturally occurring ribozymes catalyze phosphoryl transfer, a reaction that may be particularly susceptible to catalysis by metal ions. RNA can bind organic cofactors, but its ability to use them for catalysis has not been established. Whereas the ability of proteins to manipulate the electrostatic nature of the active-site environment is a key feature in the catalytic efficiency of many enzymes, RNA is not expected to be proficient at such manipulations. Finally and most fundamentally, despite the substantial differences between proteins and RNA, and the resultant inescapable differences in catalysis, the energetics of protein and RNA enzymes have an underlying commonality. Both classes of biological catalysts use binding energy from interactions away from the site of bond formation and breaking to facilitate the chemical transformation.

General Acid and Base Catalysis Vs Metal Ion Catalysis

The phosphotransesterification and hydrolysis reactions catalyzed by ribozymes require loss of a proton from the attacking hydroxyl functional group and gain of a proton on the leaving oxygen atom in the course of reaction, as shown for the *Tetrahymena* group I ribozyme reaction in Figure 2. In analogous reactions, protein enzymes often use a general base for partial removal of the proton from the attacking group, and a general acid for partial addition of a proton to the leaving group to stabilize the transition state (Figure 2, general acid-base catalysis). RNA, however, lacks functional groups with pK_as near neutrality that are optimally suited for general acid and base catalysis (Figure 1). pK_as near neutrality allow the highest concentration of the strongest acid or base to be present at physiological pH (14, 93), as can be seen for the example of general acid catalysis: Lowering the pK_a of a general acid beyond pH 7 makes the group a stronger proton donor, but more substantially decreases the amount of that group present in the functional protonated form. Increasing the pK_a of a general acid above pH 7 is also detrimental because it makes the group a weaker proton donor without significantly increasing the amount of the protonated functional group.

Do ribozymes use general acid and base catalysis with suboptimal efficiency? Can RNA control microenvironments and perturb pK_as like proteins do and thereby increase the catalytic potential of functional groups? Or do ribozymes use an alternative strategy for catalysis, such as the direct stabilization of attacking and leaving groups with metal ions (Figure 2, metal ion catalysis)?

THE TETRAHYMENA GROUP I RIBOZYME USES A METAL ION INSTEAD OF A GENERAL ACID CATALYST A series of functional groups has been substituted for the 2′-hydroxyl group adjacent to the 3′-leaving-group oxygen of the oligonucleotide substrate in the *Tetrahymena* ribozyme reaction (Figure 3). The rate of the chemical step increases substantially with the electron-withdrawing

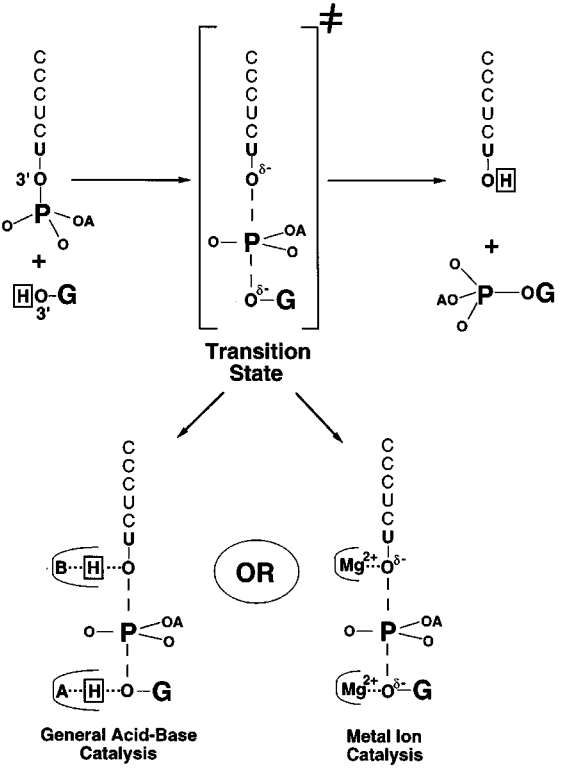


Figure 2 General acid- or base catalysis vs metal ion catalysis. The phosphotransesterification reaction catalyzed by the *Tetrahymena* group I ribozyme requires loss of a proton from the attacking 3′-hydroxyl functional group of the guanosine and gain of a proton by the leaving 3′-oxygen atom of the oligonucleotide substrate in the course of reaction. These protons are boxed.

ability of the added substituents in a manner similar to that observed in uncatalyzed solution reactions of phosphodiesters. This increase contrasts with the shallow dependence of rate on electron-withdrawing ability that is expected if a general acid provides partial protonation of the leaving-group oxygen atom in the transition state, and therefore suggests the absence of general acid catalysis (34). Instead, there is strong evidence for a direct interaction of the leaving-group oxygen atom with an active-site divalent metal ion. When the leaving-group 3′-oxygen atom is replaced by sulfur, Mg^{2+} can no longer support catalysis, but activity is restored by addition of Mn^{2+}. Because Mn^{2+} has a much greater affinity for sulfur than does Mg^{2+}, these observations suggest that the 3′-atom directly interacts with an active-site metal ion in the transition state

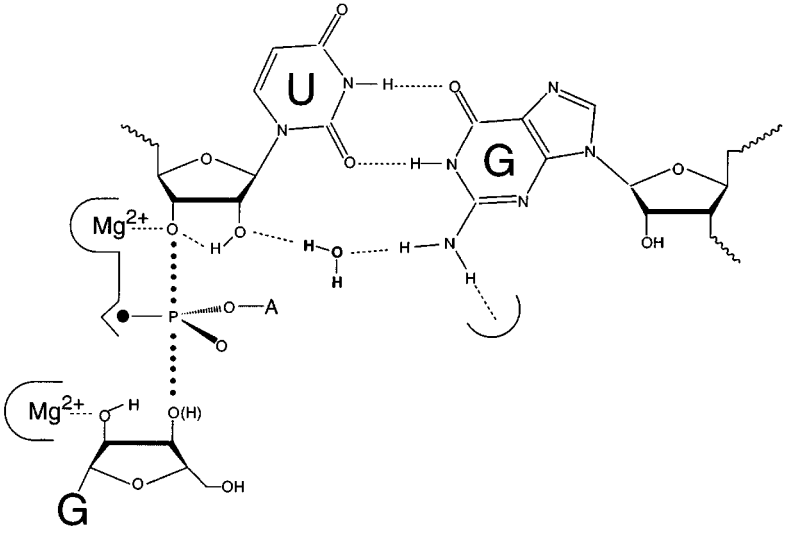


Figure 3 Transition-state interactions in the *Tetrahymena* group I ribozyme. Evidence for a direct metal ion interaction with the 3′-oxygen of the leaving group and the 2′-oxygen of the attacking guanosine comes from changes in metal ion specificity as described in the text (94; R Stromberg, personal commununication; S Shan & D Herschlag, unpublished results). Although an analogous interaction of the attacking 3′-hydroxyl group of guanosine with a metal ion is widely anticipated (53, 112), there is no direct evidence for such an interaction. An interaction between the ribozyme and the nonbridging *pro*-S_P oxygen (*filled*) is suggested from the $\sim 10^3$-fold rate reduction when this oxygen is replaced with a sulfur, in contrast to the twofold reduction in rate when sulfur is substituted at the *pro*-R_P position (238, 239). An interaction with the exocyclic amino group of the G in the G · U wobble pair was implicated from comparisons with other wobble pairs and Watson-Crick pairs at that position (39). A water molecule bridging the exocyclic amino group of G and the 2′-hydroxyl group of U was observed in the X-ray crystal structure of a duplex containing a G · U wobble pair (39a). This water molecule may make a small contribution of less than fivefold to transition-state stabilization by helping to orient the 2′-hydroxyl (52, 240).

(Figure 3) (94). Energetic aspects of catalysis arising from this metal ion are discussed below (see "Use of Intrinsic Binding Energy for Catalysis by Both RNA and Protein Enzymes").

GENERAL BASE CATALYSIS BY MG(OH)$^+$? A wide array of metal ions can support activity of the hammerhead ribozyme (Figure 4), and the efficacy of the various metal ions roughly correlates with the pK_a of their respective hydrates [i.e. $M(OH_2)_n^{2+} \rightleftharpoons M(OH)(OH_2)_{n-1}^{+} + H^+$]. A model in which a metal-hydroxide species acts as a general base was therefore suggested (95). However, the data show a correlation of rate with the ability to stabilize a bound negatively charged

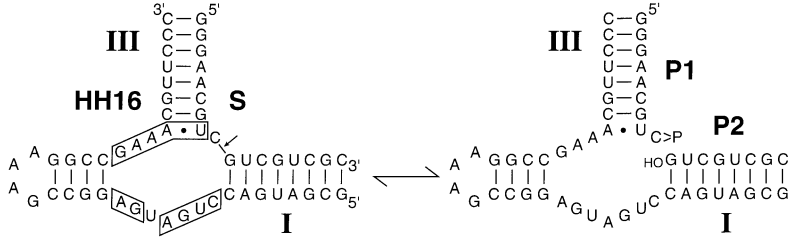


Figure 4 The reaction of the hammerhead ribozyme (27). The ribozyme binds its oligonucleotide substrate, S, via eight base pairs on either side of the cleavage site for this particular version of the hammerhead ribozyme (HH16). The boxed residues are conserved. Cleavage results in a 5′-product, P1, with a 2′,3′-cyclic phosphate product and a 3′-product, P2, with a 5′-hydroxyl group.

hydroxide ion, not the direct involvement of a metal-hydroxide species. This correlation could instead arise from mechanisms in which a metal ion interacts directly with the 2′-oxygen nucleophile, with a nonbridging phosphoryl oxygen atom, or with the 5′-oxygen leaving group, depending on the nature of the charge distribution in the transition state. In the simplest model, a direct metal ion interaction with a group that develops partial negative charge in the transition would be energetically preferred relative to an interaction through an intervening water or hydroxide, as would occur if $Mg(OH)^+$ acted as a general base catalyst. On the other hand, geometrical considerations or other electrostatic effects within the active site could favor indirect interactions.

OTHER METAL IONS IDENTIFIED IN RNA CATALYSIS A hammerhead ribozyme substrate with an R_P-phosphorothioate at the cleavage site can be efficiently cleaved in Mn^{2+} but not Mg^{2+}, providing strong evidence for a direct metal ion interaction (96, 97). Recent results also provide strong evidence for a direct interaction of a metal ion with the 2′-hydroxyl group of the nucleophilic guanosine in group I introns (Figure 3): Self-splicing of the bacteriophage T4 nrdB group I intron with 2′-amino guanosine is slow in the presence of Mg^{2+} but is accelerated considerably by the addition of Mn^{2+} or Zn^{2+}, metal ions that have greater affinity for nitrogen than Mg^{2+} (R Stromberg, personal communication). It has also been shown that Mn^{2+} increases binding of 2′-amino guanosine but not guanosine, using the *Tetrahymena* ribozyme (S Shan & D Herschlag, unpublished results).

Phosphorothioate substitutions have been used to identify phosphates on group I, RNase P, and hammerhead ribozymes that are important for function. A subset of these defects can be rescued by the addition of Mn^{2+}, suggesting direct metal ion coordination at these positions, although the role of these groups in catalysis has not been established (98–102; see also 103).

Specific metal ion interactions have also been suggested for several ribozymes based on decreases in apparent metal ion affinity following removal of a particular functional group. Although such effects can arise from removal of a group that interacts directly with a metal ion, they can also arise from indirect structural or kinetic effects. Indeed, increasing metal ion concentration typically increases the stability of folded RNAs and therefore can restore function to RNAs that contain a wide array of structural defects (e.g. 104–111), so interpretations invoking direct interactions should be viewed with considerable skepticism.

WHY ARE METAL IONS USED IN RIBOZYME CATALYSIS? *Is RNA particularly suited for using metal ions in catalysis?* The apparent consensus in the RNA community is that ribozymes generally or exclusively use catalytic metal ions (53, 112–115). However, assigning specific catalytic roles to metal ions in RNA catalysis is difficult because metal ions are essential for folding into the catalytic conformation (e.g. 116–121). This difficulty is compounded because the charged phosphodiester backbone of RNA is coated with metal ions. For example, it has been estimated that the ~400-residue catalytic RNA from *Bacillus subtilis* RNase P binds ~100 Mg ions under catalytic conditions (121). Proteins, by contrast, typically contain only a small number of well-defined metal binding sites.

Although coating RNA with metal ions makes it hard for the investigator to unravel the role of individual metal ions, localizing metal ions along the charged phosphodiester backbone may increase the chance that an RNA will position one or more of these metal ions appropriately for catalysis. Each residue in RNA contains several potential metal ligands: the nonbridging phosphoryl oxygens and the 2′-hydroxyl group of the backbone, and nitrogens and oxygens of the purine or pyrimidine base (Figure 1). The localization of metal ions to RNA in proximity to a plethora of ligands generates many local binding sites and opportunities to form a catalytic site. In contrast, proteins have no formal charge on their backbone and only a subset of their side chains are potential metal ligands (Figure 1); metal binding sites in proteins arise from the careful placement of ligands within the context of the overall tertiary structure.

The multidentate nature of metal ion coordination may make it easier for RNA to position metal ions than to position functional groups that can act as general acids and bases. In addition, the positional requirements for electrostatic stabilization of the transition state may be less stringent than for stabilization via partial proton donation or abstraction.

Are phosphoryl transfer reactions especially susceptible to metal ion catalysis? All of the known, naturally occurring ribozymes catalyze phosphoryl transfer reactions. This could be because RNA is particularly suited for using metal ions, which are in turn effective in catalysis of phosphoryl transfer. Substantial

catalysis of phosphoryl transfer can be achieved solely from well-positioned metal ions in model systems (122–125), and most protein enzymes that catalyze phosphoryl transfer use metal ions (13, 126).[3] Alternatively, the preferential or exclusive catalysis of phosphoryl transfer and the use of metal ions could have arisen because ribozymes have had sufficient selective pressure to catalyze phosphoryl transfer, a reaction that appears to be inherently suited to metal ion catalysis.

CAN RNA PERTURB pK_as TO OPTIMIZE GENERAL ACID-BASE CATALYSIS? It has been suggested that folded RNA can perturb pK_a values for functional groups toward neutrality and thereby enhance their ability to perform general acid-base catalysis (132, 133). Perturbed pK_as are the rule rather than the exception in folded RNA; the pK_as of hydrogen bond donors are raised and those of the hydrogen bond acceptors are lowered every time a base pair is formed. The cytosine residue of an oligonucleotide has the pK_a of its N3 nitrogen increased from 4.5 to ~6 by tertiary interactions with the *Tetrahymena* ribozyme that favor formation of a $G \cdot C^+$ wobble pair rather than a standard Watson-Crick base pair (52). However, groups with pK_a values perturbed in this way are not effective general acids and bases because the proton and lone pair are sequestered by the interaction.

An alternative way for RNA to perturb pK_a values is via the local environment. Positioning of charged groups has dramatic effects on pK_as in proteins. The classic example is the active site Lys of acetoacetate decarboxylase, which has its pK_a perturbed ~4 units to a value of 6 by a nearby Lys residue (134). RNA might be able to perturb pK_as of sugar and base functional groups by positioning them in proximity to a negatively charged phosphoryl oxygen atom or a positively charged bound metal ion. RNA has, at least in one instance, been shown to be highly effective in active site positioning (54) (see "Use of Intrinsic Binding Energy for Catalysis by Both RNA and Protein Enzymes"). Proteins can accentuate electrostatic perturbations by creating local environments of low effective dielectric. However, we suggest that RNA has significant limitations in this respect (see "Can RNA Manipulate the Environment of Binding Sites to Facilitate Catalysis?").

[3]Magnesium ion, which is typically used by RNA enzymes and often used by protein enzymes, tends to be less effective than other divalent metal ions in model reactions. This could be because magnesium ion is less effective at stabilizing negative charge, as shown by the high pK_a of $Mg(OH_2)$ relative to other metal hydrates (127), and because it is more difficult to bind Mg^{2+} tightly and specifically in small model systems. Also of note is that proteins can create very tight binding sites that allow the use of metal ions other than the most abundant Mg^{2+} ion. For example, carbonic anhydrase has an affinity for its catalytic Zn^{2+} ions in the low picomolar range (128). Although RNA has metal binding sites that preferentially bind Mn^{2+} and Zn^{2+} relative to Mg^{2+}, the strongest measured affinities we are aware of are in the low micromolar range (129, 130), whereas the physiological concentration of Mn^{2+} and Zn^{2+} is in the low nanomolar range (131).

SUMMARY If RNA is to use general acid or base catalysis, it may need to resort to using groups with suboptimal pK_as. This may not be a problem for catalyzing phosphoryl transfer and other reactions that are effectively catalyzed by metal ions, because RNA appears to be particularly adept at metal ion catalysis, but it may limit the repertoire of reactions that RNA can effectively catalyze. Selection experiments may help test this initial view. For example, would in vitro selections for reactions that are not as susceptible to metal ion catalysis give RNAs that utilize general acid or base catalysis? Would performing selections at pHs nearer to the pK_as of RNA side chains favor the recovery of RNA catalysts that use general acid or base catalysis? Or are there additional problems with the use of general acid-base catalysis by RNA? Finally, structures of RNA at the atomic level, especially of RNA active sites, will help us understand how RNA uses metal ions and other functionalities in catalysis.

Covalent Catalysis

In covalent catalysis, the substrate is transiently modified via covalent bond formation with an enzymatic functional group to give a reactive intermediate. Formation of a covalent intermediate allows a single step with a large activation barrier to be broken down into two steps that each have a smaller activation barrier. Covalent catalysis can be divided into three mechanistic classes on the basis of examples from protein enzymes.

CLASS 1 The enzymatic nucleophile is similar in kind and reactivity to the ultimate solution acceptor. Examples of this class include the serine proteases and the alkaline phosphatases (14, 135). The serine hydroxyl group is similar in chemical reactivity to the hydroxyl group of water, the final acceptor in these group transfer reactions. The advantage of this form of covalent catalysis presumably arises not from the chemical nature of the enzymatic group, but rather from the relative ease of positioning a nucleophile that is part of the protein itself and has more "handles" for interaction than a water molecule. Additionally, the serine ester intermediate is more reactive than the amide substrate, so attack by water is easier in the second step. Nevertheless, other enzymes catalyze these reactions as direct transfers without the use of an intervening serine nucleophile.

CLASS 2 The enzymatic nucleophile is intrinsically more reactive than the ultimate acceptor. Several enzymes use the imidazole side chain of histidine as a transient acceptor in phosphoryl transfer reactions (14). Amines react faster than alcohols with phosphoryl compounds, yet they form less stable adducts that can therefore readily allow rapid turnover (136).

CLASS 3 Formation of a covalent adduct with an enzymatic nucleophile increases the reactivity of the substrate, which facilitates a reaction that is distinct

from adduct formation. Examples of this include the thymidylate synthase reaction, in which the sulfhydryl of an active-site cysteine adds to C6 of the uracil. This breaks the aromaticity and adds electron density to increase the nucleophilicity of C5, thereby facilitating transfer of a methylene equivalent from tetrahydrofolate (137). Analogously, lysine residues form Schiff base intermediates with substrates, providing an electron sink in several enzymatic reactions (138).

The first engineered multiple turnover ribozyme derived from the *Tetrahymena* group I intron used Class-1 covalent catalysis. The 3′-hydroxyl of the terminal guanosine residue acts as an intermediary acceptor prior to the ultimate transfer of the nucleotide to water in a hydrolysis reaction or to an oligonucleotide in a phosphotransesterification reaction (16). However, unlike the serine protease reaction, the ribozyme-bound intermediate is not more reactive than the substrate. Guanosine also has more handles than a water molecule, and guanosine bound from solution reacts in the chemical step at the same rate as guanosine that is covalently attached to the ribozyme (31).

Protein enzymes greatly expand their catalytic repertoire by the use of covalent catalysis, especially Class 3. Although RNA can use Class-1 covalent catalysis, at least nominally, it lacks the reactive side chains that facilitate Class-2 and Class-3 covalent catalysis. For example, the amino groups of adenine and cytidine are unreactive relative to aliphatic amines such as lysine because of aromaticity. Protein enzymes also expand their catalytic repertoire by the use of cofactors. The ability of RNA to use cofactors is discussed in the next section.

Cofactors in Enzymatic Reactions: Can RNA Use Them Too?

Protein enzymes use an array of organic cofactors to broaden the metabolic capabilities of living systems. For example, NAD and FAD allow redox chemistry, biotin allows carboxylation, B12 and folate allow methyl and other C1 transfer reactions, and pyridoxal phosphate facilitates an array of reactions in amino acid metabolism.

In vitro selection experiments have shown that RNA can bind a vast array of ligands, including several cofactors (e.g. 139–149). Recent results suggest that binding sites for small ligands can be engineered by creating defects within structured RNAs (150). Introduction of abasic residues or base mispairs within the conserved core of the hammerhead ribozyme created low-affinity noncovalent binding sites for the base that had been eliminated at 4 of the 13 positions tested. One of the sites created by removing a guanine base could also bind pterin, the heterocycle of the folate cofactor.

Although binding is a prerequisite for use of a cofactor by an RNA enzyme, it is not sufficient for catalysis. Efficient catalysis requires both establishing

a local environment that is conducive to the reaction in question and holding the substrate(s) and cofactor in an appropriate orientation with respect to one another. The ability of RNA to control the environment within binding sites is discussed in the next section, and the ability of RNA to position substrates and catalytic groups is discussed in a subsequent section ("Why Are Protein Enzymes Big and (Some) RNA Enzymes Even Bigger? The Importance of Being Rigid"). Based on these analyses, we expect that RNA will be considerably less efficient than proteins in the utilization of cofactors, an expectation that will presumably be tested by in vitro selections in the coming years.

Can RNA Manipulate the Environment of Binding Sites to Facilitate Catalysis?

As mentioned above, we expect RNA will not be proficient at perturbing pK_a values of functional groups in order to optimize general acid and base catalysis. This expectation is based on a view that folding limitations of RNA limit its ability to control the properties of local environments in binding sites. An inability to control local environments would reduce RNA's ability to catalyze many types of reactions. The term "effective dielectric," used below, provides a convenient though imprecise way to describe some aspects of this control. The effective dielectric reflects the ability of an environment to respond to changes in charge and lessen the energetic consequences of the change; this response requires the presence of and movement of polar groups.

For example, it has been suggested that perturbations of the active-site environment relative to aqueous solution are used by proteins to increase the energetic contribution to catalysis from hydrogen bonds between the reactant and active site (151–155). This can be accomplished most simply if proteins control their environment to create active sites with effective dielectric constants lower than that in solution (see below). The lower effective dielectric can result in a larger change in hydrogen bond strength at the active site than in aqueous solution. Thus, an increase in hydrogen bond strength as charge redistributes in going from the ground state to the transition state can provide catalysis for the enzymatic reaction relative to the reaction in solution (155). Increasing the sensitivity of hydrogen bond strength to charge rearrangements may be a general catalytic strategy because nearly every biological reaction involves charge redistribution at heteroatoms.[4] Furthermore, the increased sensitivity of hydrogen bond strength in a low dielectric medium follows simply from Coulomb's law, so

[4]Proteins can also provide larger increases in hydrogen bond strength in the course of a reaction by using side chains that are stronger hydrogen-bond donors and acceptors than water (34, 156, 157). Protein side chains contain stronger hydrogen-bond donors and acceptors than the RNA side chains, and even the charged oxygens of the RNA's phosphodiester backbone are relatively weak hydrogen-bond acceptors, as indicated by their low pK_a of $\sim$1.

an analogous increase in sensitivity is predicted for all electrostatic interactions [discussion of these issues is beyond the scope of this review; see (154, 155)].

The following structural considerations suggest that RNA is limited in its ability to fix or control local environments, an ability required to exploit the catalytic strategies described above. The hydrophobic side chains and hydrophobic core of proteins contribute, of course, to the creation of a low dielectric environment. Furthermore, even though proteins have polar and charged groups in addition to their hydrophobic side chains, a low effective dielectric is maintained because the motions of the polar and charged groups are greatly restricted within a precisely packed three-dimensional structure (158, 159). Such restricted motion allows maximal stabilization of the transition state relative to the ground state, as is required for catalysis (see "Why Are Protein Enzymes Big and (Some) RNA Enzymes Even Bigger? The Importance of Being Rigid").

RNA has a more difficult job than proteins in creating a low effective dielectric environment. RNA lacks side chains that are purely hydrophobic, making it difficult for RNA to create an extensive hydrophobic pocket of low dielectric. Whereas most protein side chains are hydrophobic, each RNA side chain has a dipole that can potentially rearrange to increase the effective dielectric. Thus, RNA must provide more structural interactions than proteins in order to achieve a comparable low effective dielectric. However, the following considerations suggest that RNA is substantially less adept than proteins at making structural interactions that can fix groups within an active site. The severely limited diversity of the RNA side chains relative to protein side chains, and the charge on the RNA backbone (Figure 1), will limit close packing. Without close packing, rearrangements of the polar bases and the charged backbone are facile. The absence of tight packing also makes it difficult to prevent access of bulk or highly mobile water, which can further increase the local dielectric. Finally, the greater number of degrees of freedom within the RNA backbone compared to the protein backbone (Figure 1) is expected to increase structural plasticity of the charged and dipolar groups of the backbone and of the dipolar side chains, thereby increasing the local dielectric.

Use of Intrinsic Binding Energy for Catalysis by Both RNA and Protein Enzymes

According to transition-state theory, catalysis requires, and indeed can be defined as, stabilization of a reaction's transition state without equivalent stabilization of the ground state (160, 161). In 1975, Jencks presented a comprehensive analysis of the energetics of enzymatic catalysis (1). He noted that many enzymes respond to the addition of a substrate functional group with an increase in k_{cat} instead of an increase in binding affinity, even though the added functional group was remote from the site of chemical transformation. These and other observations led to the concept of intrinsic binding energy. According to

this concept, the energy from binding interactions is expressed in the transition state but not in the ground state of the enzyme · substrate complex.

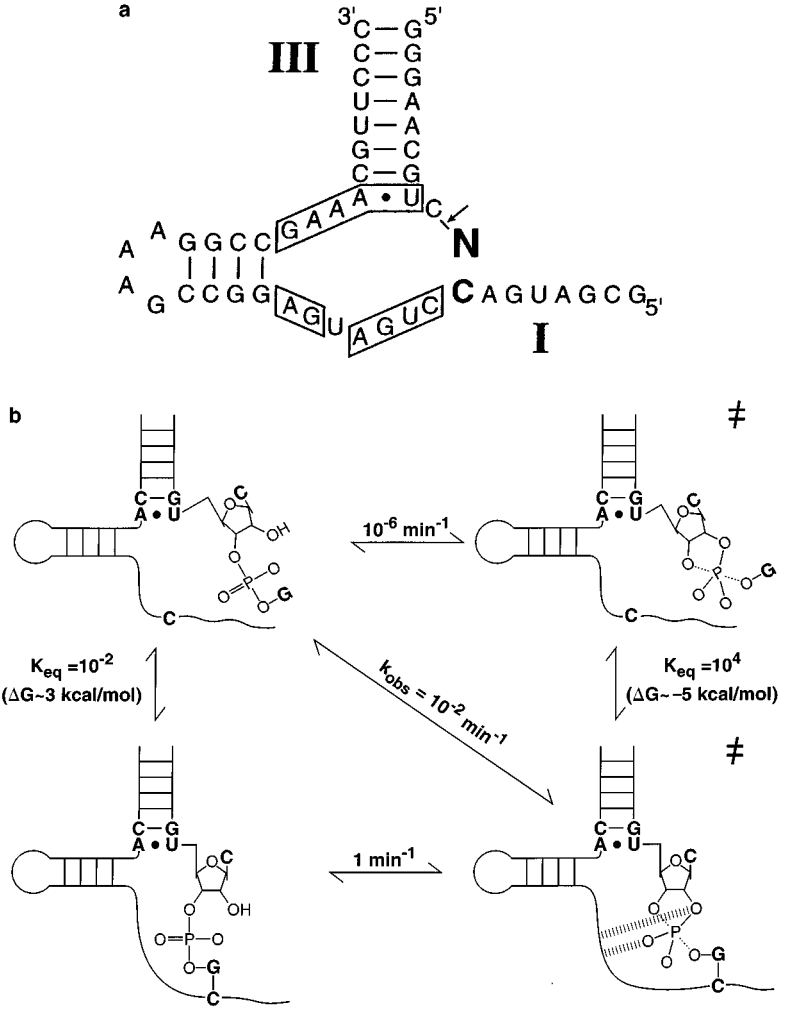
The binding energy can be used to position one substrate with respect to another, or to position a substrate with respect to catalytic groups on the enzyme. Thus, the bound substrates can be considered to be entropically destabilized relative to solution so that the binding energy used for positioning is not expressed in the free energy of the ground-state complex. However, the substrates are now prepositioned for reaction, facilitating reaction of the bound complex (162). There is also evidence that binding energy can be used to position the reactive portions of a substrate in an environment that is destabilizing relative to aqueous solution, but is better suited to stabilizing the transition state (1). This also facilitates reaction of the bound complex. In both cases, the intrinsic binding energy is expressed in the transition state. The observed binding energy is often less than the intrinsic value, the energy that would be obtained in the absence of a loss of entropy and destabilizing interactions.

This concept of intrinsic binding energy and the use of binding interactions away from the site of bond formation and breaking to facilitate the reaction has provided an energetic framework for analyzing and understanding enzymatic catalysis. The discussion below describes the use of intrinsic binding energy in two RNA enzymes, the hammerhead ribozyme and the *Tetrahymena* group I ribozyme. This extends this energetic principle to RNA enzymes and suggests that the use of binding energy is a general feature of biological catalysis.

INTRINSIC BINDING ENERGY IN CATALYSIS BY THE HAMMERHEAD RIBOZYME

Hammerhead ribozymes contain two recognition arms whose sequences are not conserved. The recognition arms form Watson-Crick helices with oligonucleotide substrates on either side of the substrate's cleavage site (Figure 4). Cleavage of oligonucleotides that form only one of the two helices with the ribozyme is not catalyzed: When bound to the ribozyme via one of the recognition arms, the reaction occurs at the same rate as the background reaction in solution, which is 10^6-fold slower than the catalyzed reaction (163; K Hertel, A Peracchi, OC Uhlenbeck & D Herschlag, in preparation). However, replacing one residue of the oligonucleotide to allow formation of a single Watson-Crick base pair with the second recognition arm immediately adjacent to the central conserved core increases the rate of the chemical step 10^4-fold above background (Figure 5*a*) (K Hertel, A Peracchi, OC Uhlenbeck & D Herschlag, in preparation). Despite the large transition state effect of this base pair, it does not contribute to binding of the oligonucleotide. These results parallel those obtained with protein enzymes, in which groups not directly involved in the chemical transformation contribute to chemistry rather than binding (1). Thus, the hammerhead RNA enzyme can, like protein enzymes, use binding energy for catalysis.

The energetic contributions of this base pair are summarized in the model of Figure 5*b* (K Hertel, A Peracchi, OC Uhlenbeck & D Herschlag, in preparation). The maximal free energy of base-pair formation of about -3 kcal mol^{-1} (164) is much less than the $\sim$6 kcal mol^{-1} required to close the conserved core (27; T Stage, K Hertel & OC Uhlenbeck, personal communication). Thus, base-pair formation is unfavorable, by $\sim$3 kcal mol^{-1}, allowing only transient formation in the ground state. However, the base pair can contribute more in the transition state (Figure 5*b*; $\Delta G = -5$ kcal mol^{-1}). A considerable amount of the intrinsic binding energy of base-pair formation is unavailable in the ground state because



entropy is lost from fixation of the residues within the base pair. In the transition state, this binding energy can be expressed because the lost entropy can be used to position the reactive phosphoryl group of the substrate with respect to the conserved catalytic core, thereby contributing to catalysis. Indeed, the energy from this base pair is estimated to be $\sim$8 kcal mol^{-1} more in the transition state than in the ground state (Figure 5*b*).

INTRINSIC BINDING ENERGY IN CATALYSIS BY THE TETRAHYMENA GROUP I RIBOZYME The oligonucleotide substrate of the *Tetrahymena* ribozyme binds in two steps (Figure 6*a*). In the first step, the substrate base-pairs to a region of the ribozyme referred to as the internal guide sequence, forming the P1 helix in the open complex. In the second step, the P1 helix docks via tertiary interactions, forming the closed complex (48, 50, 165–167). The functional groups of the P1 duplex that contribute to these tertiary interactions have been identified, and their energetic contributions to duplex formation and tertiary stabilization have been dissected (33, 38, 39, 51, 52, 168–170). The sum of the tertiary stabilization provided by the individual groups is 11 kcal mol^{-1} (Figure 6*b*) (33, 38, 51, 52, 170). However, the observed tertiary stabilization is only 2 kcal mol^{-1} (54, 170). This discrepancy is not due simply to nonadditive binding interactions, because at least 4 kcal mol^{-1} of the sum comes from tertiary interactions that are energetically additive (38, 170). Instead, there appears to be an energetic barrier for docking of the P1 duplex into the active site that is paid for by the intrinsic binding energy ($\Delta G_{intrinsic}$) of functional groups on the P1 duplex (Figure 6*c*). Comparisons of the binding and reactivity of a series of oligonucleotide substrates and products suggest that the energetic barrier for P1 docking results from the cost of positioning the substrate within the active site (ΔG_{posn}) and electrostatically destabilizing the substrate (ΔG_{destab}) (Figure 6*c*) (54). The observed tertiary stabilization (ΔG_{obs}) of 2 kcal mol^{-1} is what remains

Figure 5 Use of intrinsic binding energy by the hammerhead ribozyme (K Hertel, A Peracchi, OC Uhlenbeck & D Herschlag, in preparation). (*a*) The hammerhead ribozyme, HH16, complexed with a substrate that base-pairs to make helix III but not helix I (see Figure 4). The identity of the nucleotide, N, directly adjacent to the cleavage site was systematically changed as described in the text. (*b*) The base pair directly adjacent to the cleavage site specifically stabilizes the transition state. Binding measurements indicate that the G · C base pair is not formed in the ground state. In the presence of a mismatch at the site, the cleavage rate constant is not enhanced beyond the background rate constant of 10^{-6} min^{-1}. In the presence of a G, which allows formation of the G · C base pair, cleavage is observed at 10^{-2} min^{-1}. The full-length substrate, which can form both helix I and helix III, is cleaved with a rate constant of 1 min^{-1}. Formation of the G · C base pair in the ground state is unfavorable by $\sim 10^2$-fold. In contrast, the equilibrium constant for forming the G · C base pair in the transition state ($\neq$), as obtained from the other rate and equilibrium constants in the figure, is favored by 10^4-fold. This difference of 10^6 corresponds to an 8 kcal mol^{-1} greater stabilization of the G · C base pair in the transition state than in the ground state.

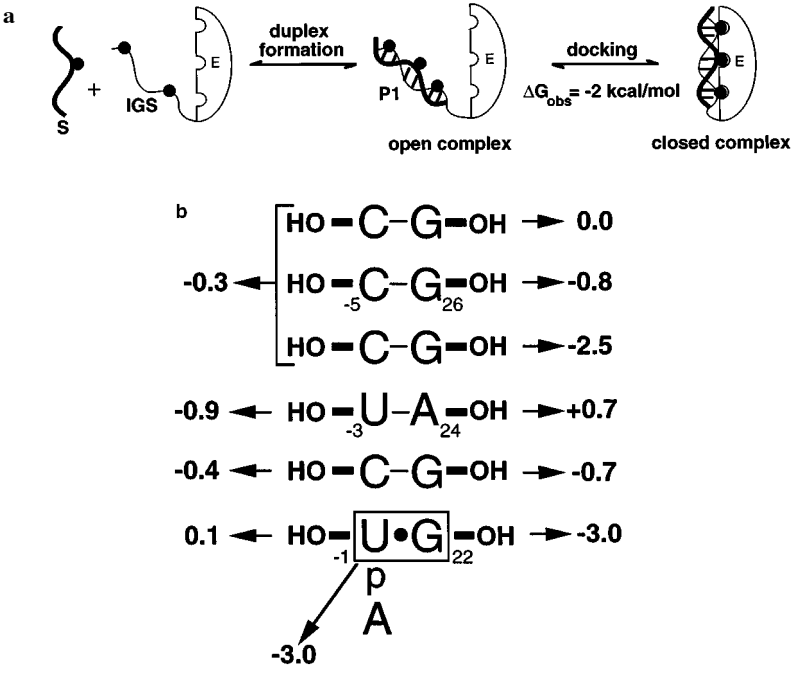


Figure 6 Binding of the *Tetrahymena* ribozyme's oligonucleotide substrate. (*a*) Two-step binding of the substrate. S refers to the oligonucleotide substrate, IGS refers to the *Tetrahymena* ribozyme's internal guide sequence, and E refers to the ribozyme. Groups involved in tertiary interactions are shown schematically as filled circles. (*b*) The tertiary binding energies provided by individual groups on the P1 duplex formed between the ribozyme's internal guide sequence (5′-GGAGGG) and substrate (5′-CCCUCUA). The sum of individual tertiary energies is -11.0 kcal mol^{-1}. (*c*) Use of intrinsic binding energy for positioning and substrate destabilization. Abbreviations and symbols are as defined in (*a*). The contribution of particular interactions to $\Delta G_{intrinsic}$ cannot be discretely separated into ΔG_{posn} and ΔG_{destab} because positioning is required for inducing substrate destabilization. ΔG_{destab} equals $\sim$2 kcal mol^{-1}, but the size of the barrier to positioning, ΔG_{posn}, is not known, because the extent of independence of the individual binding interactions is not known (170).

after the intrinsic binding energy of tertiary interactions is utilized to overcome this energetic barrier [Figure 6*c*; $\Delta G_{obs} = \Delta G_{instrinsic} - (\Delta G_{posn} + \Delta G_{destab})$].

The open and closed complexes of the *Tetrahymena* ribozyme (Figure 6*a*) can be distinguished kinetically, energetically and structurally, and the transition between these forms is well described by a discrete two-state process (48, 165–167). This renders assessment of the expression of intrinsic binding energy in binding and catalysis straightforward. These and other observations

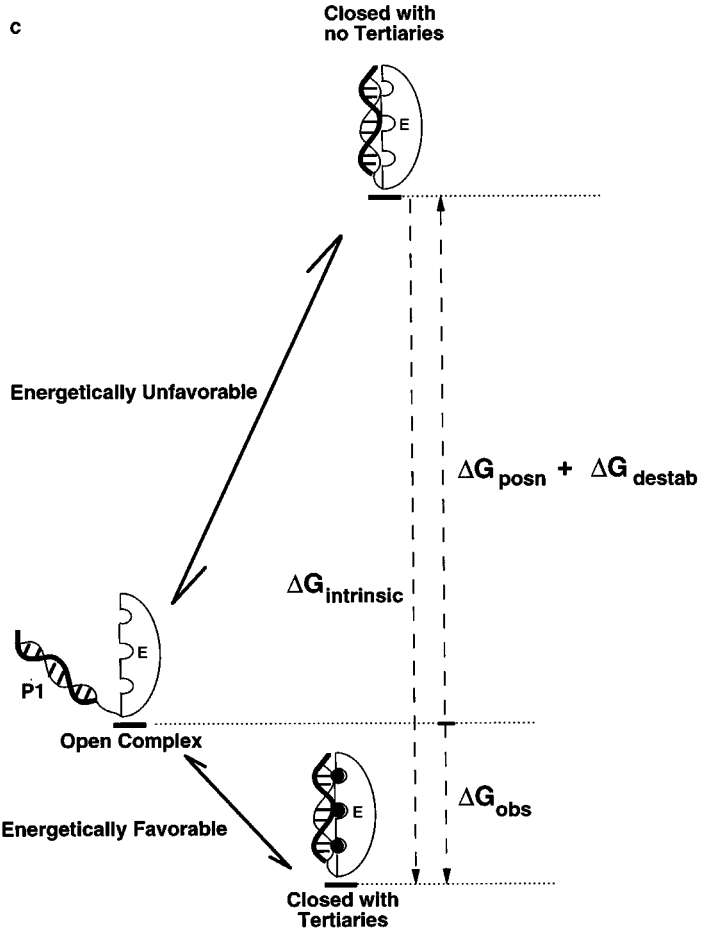


Figure 6 (*Continued*)

suggest that RNA enzymes may be particularly amenable to energetic dissection and mechanistic analysis. Below we describe the mechanisms by which the *Tetrahymena* ribozyme uses intrinsic binding energy for catalysis.

Use of intrinsic binding energy to position substrates within an RNA active site Proteins use binding energy to precisely position substrates within active sites (1). But positioning requires rigidity, and as noted elsewhere, RNA is expected to have difficulty creating rigid binding sites because of the limited diversity of its side chains and the flexibility and charge of its backbone (Figure 1).

Despite these limitations, there is evidence that the *Tetrahymena* ribozyme can be effective in positioning functional groups (54). The 3′-hydroxyl group of the oligonucleotide product interacts with a Mg^{2+} ion in the active site (Figure 3). Removal of this hydroxyl group and replacement by a 3′-hydrogen atom weakens tertiary binding 40-fold. Thus, compared to 55 M water, the 3′-hydroxyl of the bound product has a 40-fold stronger interaction with the Mg^{2+} ion, even though the hydroxyl group and competing water are electrostatically similar. This corresponds to an "effective concentration" of this hydroxyl group at the RNA active site of 40 × 55 M or 2200 M. This large effective concentration rivals those observed with protein enzymes of 10^3–10^5 M (1, 171–173).[5]

Given the apparent difficulties of creating a fixed or rigid active site made up of RNA, how does the *Tetrahymena* ribozyme achieve the precise positioning required for such a large effective concentration? We can identify three factors that can contribute to the ability of this RNA to attain precise positioning: 1. Rigidity from duplex formation. An oligonucleotide substrate, $UCUA_5$, lacks three of the base-pairing residues of the P1 helix but retains all of the 2′-hydroxyl groups that contribute tertiary binding energy. Nevertheless, the tertiary binding energy for this substrate is reduced by $\sim 10^2$-fold (GJ Narlikar, M Khosla & D Herschlag, in preparation). This suggests that base-pair and duplex formation helps to reduce the conformational entropy and to position 2′-hydroxyl groups for tertiary interactions. 2. Rigidity from helix/helix interactions. The structure of the P4-P6 domain of the *Tetrahymena* ribozyme provides the first direct demonstration of helix/helix packing in RNA (85, 177). Interactions alongside the P1 duplex can presumably further reduce the flexibility of the duplex (see also "Why Are Protein Enzymes Big and (Some) RNA Enzymes Even Bigger? The Importance of Being Rigid"). 3. Rigidity from metal ion coordination. Metal ions can bring together an array of functional groups via coordination. This can increase the precision of positioning within an RNA structure. Thus, high effective concentrations achieved by RNA may require the direct or indirect involvement of metal ions.

Understanding how RNA is able to achieve high effective concentrations will require direct structural determination of the networks of interactions that are responsible, along with further functional studies to resolve the energetic contributions of these structural elements.

[5]Effective concentrations that arise from entropic factors can far exceed concentrations that are attainable in practice. This is because much entropy remains in two molecules that are next to one another in solution relative to the entropy lost when these two molecules are fixed with respect to one another by formation of a covalent attachment. The maximal effective concentrations from such fixation are $\sim 10^8$ M (162, 174–176).

Use of intrinsic binding energy to facilitate ground-state destabilization within an RNA active site The oligonucleotide substrate of the *Tetrahymena* ribozyme reaction, CCCUCUpA, contains the reactive phosphoryl group, and substitutions of the nonbridging oxygen atoms of this phosphoryl with sulfur suggest that the ribozyme interacts with the *pro*-S_P oxygen atom (Figure 3). It was therefore surprising to observe 40-fold greater tertiary stabilization for binding of the oligonucleotide product, $CCCUCU_{OH}$, which lacks the reactive phosphoryl group (54).

As mentioned above, there is strong evidence for a direct interaction between a Mg^{2+} ion and the 3′-leaving group oxygen atom of the substrate in the transition state (94; Figure 3). An interaction between this Mg^{2+} and the oxygen atom in the ground state can account for the paradoxical destabilization of the bound substrate. This is because the phosphoryl group is electron withdrawing, so an electrostatic interaction of Mg^{2+} with the 3′-oxygen of the substrate is expected to be weakened relative to an interaction with the 3′-oxygen of the product or with the oxygen of water (Figure 7*a*). Thus, this interaction would not be expected in solution. However, tertiary binding interactions force this destabilizing interaction by positioning the 3′-bridging oxygen of the substrate next to the Mg^{2+} ion. In contrast to this electrostatic destabilization in the ground state, the interaction becomes stabilizing in the transition state (relative to an interaction with water) because the bridge oxygen carries a partial negative charge (Figure 7*a*, $\neq$). These strategies of substrate destabilization and transition-state stabilization provide estimated rate enhancements of 300-fold and 60-fold, respectively, accounting for $\sim 10^4$ of the overall 10^{11}-fold rate enhancement achieved by the *Tetrahymena* ribozyme (Figure 7*b*) (54).

Analogous destabilization is expected whenever a metal ion or hydrogen bond donor are positioned next to the bridge oxygen of a reactive phosphate group. Such an interaction with the 3′-oxygen of the guanosine nucleophile in the *Tetrahymena* ribozyme reaction may be responsible for the ~100-fold destabilization of GpA binding relative to guanosine (31, 178; TS McConnell, D Herschlag & TR Cech, unpublished results). Substrate destabilization is a potential mechanism for protein enzymes such as the 3′–5′-exonuclease of DNA Pol I, DNase I, and alkaline phosphatase, which appear to have the reactive bridging oxygen positioned next to a metal ion that presumably stabilizes the oxyanionic leaving group in the transition state (179–181). Staphylococcal nuclease, G proteins, and fructose-1,6-biphosphatase appear to have hydrogen bond donors near the reactive bridging oxygen that could provide analogous substrate destabilization and transition-state stabilization (182–185). The bridging oxygen of carbon esters and of glycosides is also electron deficient relative to

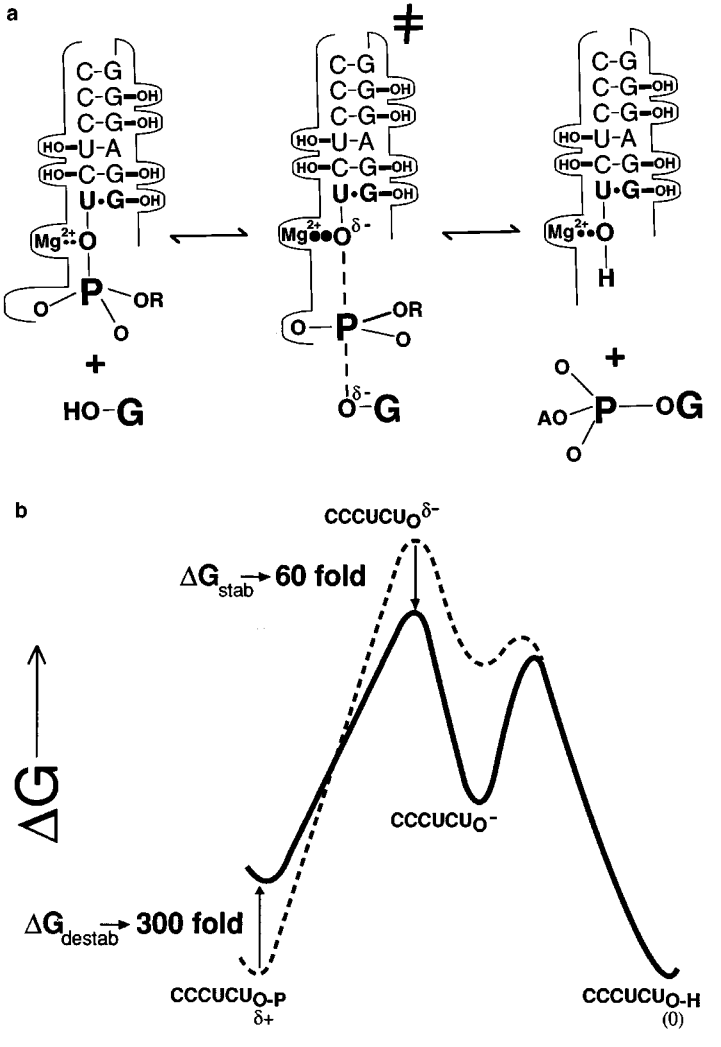


Figure 7 Ground-state destabilization and transition-state stabilization by electrostatic interactions with the *Tetrahymena* ribozyme (54, 94). (*a*) Model for the use of binding energy to induce electrostatic substrate destabilization. The ribozyme is represented schematically by the thin outline. The sequences of the internal guide sequence (5′-GGAGGG) and substrate (5′-CCCUCUA) are shown explicitly. The strength of the electrostatic interaction is indicated by the size of the dots. (*b*) A hypothetical free-energy profile in the absence (*dashed line*) and presence (*solid line*) of the active site Mg^{2+} showing the estimated rate enhancement achieved by substrate destabilization and transition-state stabilization. The free-energy changes are depicted relative to the 3′-hydroxyl of the oligonucleotide product, which is electrostatically similar to a water molecule. Thus, the values represent estimates of the electrostatic effects relative to those with water, and catalysis relative to the reaction in aqueous solution.

a protonated oxygen, so enzymes catalyzing carbon ester hydrolysis could use analogous substrate destabilization (185a, 185b).

Why Are Protein Enzymes Big and (Some) RNA Enzymes Even Bigger? The Importance of Being Rigid

As noted above, transition-state theory allows catalysis to be defined as preferential stabilization of a transition state relative to a ground state (160, 161). An enzyme must therefore be able to distinguish between the ground and transition states. A simple corollary is that a rigid enzyme will be more effective than a floppy enzyme: In the extreme, a completely floppy enzyme would not care if it were bound to the ground state or transition state; in contrast, rigidity would allow the enzyme to maximize the specificity of interactions with the transition state relative to the ground state and therefore maximize catalysis (186, 187).[6] The large size of enzymes is presumably a manifestation of the requirement to limit flexibility (see also 189–192; some of these references also discuss additional reasons for the large size of proteins). We suggest that the inherent limitations of RNA packing, relative to protein packing (Figure 1), give rise to a requirement for more RNA structural elements and a larger overall structure to achieve rigidity. The following comparisons provide support for this view.

Consider an RNA enyzme and protein enzyme that catalyze the same reaction: the hammerhead ribozyme and ribonuclease A. Despite their similar mass, RNase A achieves a 10^5-fold-larger maximal rate (K Hertel, A Peracchi, OC Uhlenbeck & D Herschlag, in preparation; 193).[7] In addition, removal of any of the side chains of residues in the conserved core of the hammerhead ribozyme, by replacement with an abasic residue, has a large effect on catalysis. Nine of the 13 positions give a reduction of more than 10^3, and 5 of these give a reduction of 10^6 and a reaction rate that is indistinguishable from the background rate (150). Removal of the side chains of His12 and His119 from RNase A results in large reductions in catalysis (193). Even though these are considered to be the catalytic residues, and despite the larger rate enhancement with RNase A, the effects from these mutations of 10^3–10^4-fold are considerably less than those observed for a large fraction of the residues in the hammerhead system.

[6]The term rigidity is not meant to imply that there are no internal motions; it is meant as a relative term and is used throughout for simplicity. In addition, there are cases in which a certain amount of flexibility can enhance catalysis (188). Though these cases may be of widespread significance, they do not affect the general points in the text. Some aspects of the importance of conformational flexibility are discussed in "Comparisons of the Ability of RNA and Protein Enzymes to Carry Out Complex Functions."

[7]We focus on structural distinctions for the purposes of this section, even though some of the difference in catalytic efficiency may arise from the limited repertoire of RNA side chains, which does not include the imidazole group that is used as a general acid and base by RNase A (see "General Acid and Base Catalysis vs Metal Ion Catalysis").

Further, protein mutagenesis experiments show that only a small fraction of the side chains typically provide a large contribution to catalysis.

These observations support the view that this small RNA enzyme barely achieves the positioning required for catalysis. This view is consistent with the low effective concentration of 10^{-2} M for the bound products of the hammerhead ribozyme (27).[5] In contrast, because of the extensive networks of interactions and interconnections within a folded protein, removal of any single side chain is unlikely to disable the active site.

The larger RNA enzymes, such as the group I ribozymes and RNase P, provide a rate enhancement of 10^{11}-fold, considerably more than the enhancement of $\sim 10^6$-fold attained by the smaller ribozymes (26, 28; K Hertel, A Peracchi, OC Uhlenbeck & D Herschlag, in preparation). We suggest that the larger size ensures the positioning and rigidity within the active site that are required for most effective catalysis. (Some of the observed difference could also arise because these ribozymes catalyze different types of reactions.) Group I ribozymes are typically over 200 residues, and the best characterized of these from *T. thermophila* is over 400 residues, corresponding to a molecular weight of ~130,000. The group-I motif is specified by a core region that is highly conserved among the more than 200 sequenced examples, but different subclasses contain different extensions from this core (194–196). These observations combined with results from structure mapping by solution-based free radical cleavage (119, 197–200) suggest a view of RNA folding that is distinct from the general view of protein folding. Whereas a central hydrophobic core can be considered the defining feature in protein folding (201), RNA folding may be ensured by the packing of domains on the outside of a central conserved region.

One of these extensions present in the *Tetrahymena* ribozyme, referred to as P5abc, is a 69-residue region consisting of several helical regions and loops. The recent X-ray structure of the P4-P6 domain of the *Tetrahymena* ribozyme reveals that P5abc lies alongside the conserved P4 and P6 regions, which are coaxially stacked, and appears to be instrumental in positioning residues in conserved regions, especially residues in nonhelical regions (85, 177).

We suggest that large extensions of RNA are generally required for positioning and fixation. This is because formation of helices and other structures is required to impart the extension with a rigidity sufficient for it to help position other regions. In contrast, because of the greater diversity of protein side chains and the limited backbone flexibility, fewer residues are required for efficient packing. In addition, the duplex rigidity may impose limitations on the local conformations of functional groups thereby further limiting close packing within RNA. Thus, the close packing within folded proteins presumably cannot be matched by RNA. Hence, we expect that proteins can achieve

substantially greater active-site rigidity, and thus more accurate positioning, than can be achieved by even the largest RNAs, though we are aware of no data that directly bear on this question. As noted above, the multidentate coordination of metal ions in RNA may provide RNA its best opportunity to create a rigid local arrangement of functional groups and on occasion match the effective concentrations obtained by proteins. The analysis of this section leads to the prediction that it should be possible to obtain, via selection, large RNA enzymes that utilize the chemical pathway of the small ribozymes while achieving substantially greater catalysis.

COMPARISONS OF THE ABILITY OF RNA AND PROTEIN ENZYMES TO CARRY OUT COMPLEX FUNCTIONS

Protein enzymes execute many complex biological functions. These include multi-step reactions, such as successive reactions that occur within a complex (e.g. fatty-acid synthesis and polyketide synthesis), and processive reactions (e.g. DNA and RNA synthesis). In addition, allosteric effectors regulate enzymatic processes. Can RNA also carry out complex functions? We first review what is known about self-splicing of group I introns, as it provides a good example of a multi-step reaction that is carried out by RNA. We then describe several additional observations that pertain to the ability of RNA to carry out complex functions.

Group I Self-Splicing

Figure 8 depicts the self-splicing pathway for group I introns. There are two chemical steps, each involving a transphosphoesterification reaction. In the first chemical step, the 3′-hydroxyl group of an exogenous guanosine cofactor (G) attacks at the phosphodiester linkage corresponding to the 5′-splice site. This breaks the covalent bond joining the 5′-exon to the intron and results in covalent attachment of the G to the 5′-end of the intron. The second chemical step is analogous to the reverse of the first step, except that a guanosine at the 3′-end of the intron (ωG), which defines the 3′-splice site, replaces the exogenous G. The 3′-hydroxyl group of the 5′-exon then attacks at the 3′-splice site to generate ligated exons and a free intron. Studies of mutant introns with G and ωG analogs have provided strong evidence that the same guanosine binding site within the conserved core is used in both chemical steps (202–204; see also 31).

To ensure efficient self-splicing, several requirements need to be met. A conformational change is required between the first and second chemical steps to allow the exogenous G to be replaced by ωG at the active site (Figure 8). There is evidence for such a conformational change from results with the group I intron from *Anabaena* tRNA (202). Golden & Cech obtained kinetic evidence for a

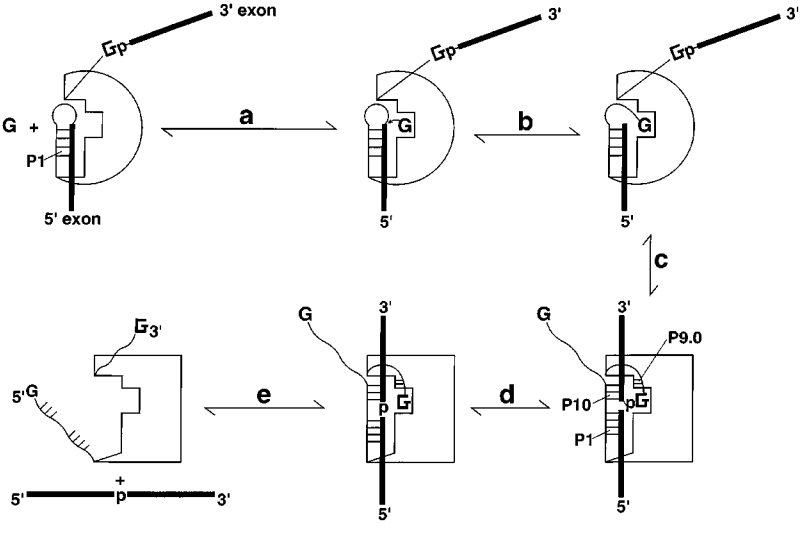


Figure 8 Steps in group I intron self-splicing. The steps are labeled "a" through "e." Steps b and d represent the first and second chemical steps, respectively. For simplicity, a single conformational step, step c, is shown between the two chemical steps and depicts the exit of the exogenous G that has become attached to the 5′-end of the intron from the active site, the entry of ωG (*square G*) into the active site, and the formation of helices P9.0 and P10; it is not known if P9.0 is formed after the first chemical step or if it is present at the initiation of the splicing reaction.

conformational step subsequent to the first chemical step that prevents reversal of that step (202). The conformational step was sensitive to mutations of ωG and mutations at the guanosine binding site, as expected for a rearrangement of bound guanosines. Recent kinetic studies of group II-intron self-splicing also suggest the presence of a conformational rearrangement separating the first and second chemical steps (205; see also 206).

The rate of chemical step in the *Tetrahymena* and *Anabaena* group I ribozyme reactions increases log-linearly with pH in the neutral region (34, 53). The absence of a pH dependence for the second step of self-splicing of the *Anabaena* intron suggests the presence of a second conformational step that intervenes between the chemical steps (AJ Zaug & TR Cech, personal communication). This conformational step could be the same as that identified from pH-dependencies for reactions of ribozymes derived from the *Tetrahymena* and *Anabaena* group I introns (25, 47).

Two paired regions, referred to as P9.0 and P10, are involved in positioning the 3′-splice site in the active site of group I introns and could be involved in conformational rearrangements between the first and second chemical steps

(203, 207–210). Indeed, some of the same residues that make up the P10 helix are involved in pairing to the 5′-splice site in the first chemical step, so a conformational rearrangement involving these residues is presumably required (207–210).

Another requirement to ensure efficient self-splicing is that the 5′-exon not be released to solution between the first and second chemical steps (36). After the first chemical step, the 5′-exon is no longer covalently attached, but it must nevertheless remain bound to ensure attachment to the 3′-exon in the second chemical step. We use calculations of base pairing energetics and results for the *Tetrahymena* intron reaction to illustrate this problem and its solution. If the 5′-exon were held in place solely by base pairing, it would dissociate with a half-time of ∼30 ms (51, 211). This is faster than the half-time of the chemical step, which is ∼1000 ms (211a), even without accounting for the time required for the conformational change(s) that form P10 and bring ωG into the active site. However, the tertiary interactions with the duplex containing the 5′-exon slow dissociation by ∼1000-fold to give an estimated half-time for dissociation of ∼30 s (36; GJ Narlikar & D Herschlag, unpublished results). This is presumably long enough to allow the conformational rearrangement and second chemical step. Thus, in addition to their catalytic role in positioning and substrate destabilization (see "Use of Intrinsic Binding Energy for Catalysis by Both RNA and Protein Enzymes"), the tertiary interactions also appear to play a biological role in preventing loss of the 5′-exon. This is analogous to the tight binding of intermediates by protein enzymes that carry out multistep reactions (212, 213).

Strong binding of the 5′-exon is necessary but not sufficient for ensuring efficient self-splicing. The tertiary interactions that slow dissociation of the 5′-exon are also expected to slow dissociation of the ligated exons, and the newly added 3′-exon of the ligated exon species can make additional base pairs to the intron (referred to as the P10 helix). This raises two problems. 1. The second chemical step (Figure 8, step "d") has been shown to be reversible with an equilibrium constant near 1 for a truncated form of the *Tetrahymena* intron (31). Thus, if all else were equal, the 5′-exon rather than the ligated exons would dissociate. This problem appears to be solved, at least in part, by reducing the kinetic barrier for dissociation of the ligated exons relative to the 5′-exon (31). 2. Even if dissociation of ligated exons can be favored, the additional base pairing with the 3′-exon of the ligated exon species is predicted to slow dissociation of the ligated exons, giving an estimated half-time of ∼4 days (31). However, recent results on splicing of the *Tetrahymena* intron show that the ligated exon species dissociates much faster than predicted, suggesting the occurrence of a destabilizing interaction that facilitates this dissociation (213a).

Processivity of an RNA Enzyme

Processivity is a hallmark of protein polymerases, certain motor proteins, and some degradative enzymes. Processivity has been demonstrated in an RNA endonuclease reaction catalyzed by a mutant form of the *Tetrahymena* group I ribozyme (165). As noted above, binding of the oligonucleotide substrate of the *Tetrahymena* ribozyme occurs in two steps (Figure 6*a*) (48, 165, 166). Docking into tertiary interactions in the second binding step positions the reactive phosphodiester in the active site, and cleavage occurs from this closed complex (33, 50). Miscleavage (i.e. cleavage at positions other than that corresponding to the 5′-splice site) occurs when the P1 duplex, containing the bound substrate, is docked into the active site in alternate registers, with different 2′-hydroxyl groups of the duplex making tertiary interactions with the ribozyme's core (Figure 6*a*) (165, 214). This finding raised the possibility that the ribozyme could cleave an oligonucleotide substrate processively, and such processivity was observed with a mutant ribozyme (165). The steps in the processive cleavage are as follows: (*a*) The substrate duplex docks normally, giving normal cleavage. (*b*) The substrate duplex undocks, giving the open complex with a shortened oligonucleotide bound. (*c*) Prior to dissolution of the duplex, the duplex redocks into an alternative binding register a significant fraction of the time to allow a second cleavage event. Use of a mutant ribozyme is necessary because it allows alternate binding registers to be sampled to a significant extent.

An Allosteric RNA Enzyme

Protein enzymes have the ability to change conformation and thus change function in response to changing conditions. Such allosteric regulation is central to the integration of information in the control of biological processes. Recently, the hammerhead ribozyme has been converted into an allosteric RNA enzyme (150). This conversion was accomplished by introducing structural defects in the ribozyme's conserved core, by removing the base (i.e. mutation to abasic residues), or by introduction of a base mispair. These defects could be functionally repaired by adding the deleted or mutated base exogenously. Thus, the added base can act as an allosteric activator of the RNA enzyme.

Summary

The above examples demonstrate the principle that RNA has the ability to effect conformational rearrangements to enhance function. Conformational rearrangements of RNA are also involved in the more complex cellular processes of pre-mRNA splicing by the spliceosome and protein synthesis by the ribosome (215–218). These processes may employ RNA because RNA is good enough to meet cellular requirements. Alternatively, RNA may have been used early in

the evolution of these complexes, and later in evolution it may have been difficult to replace all of the RNA components with proteins without disrupting the rest of the assembly. A final possibility is that RNA is inherently well suited for carrying out these processes, especially as these processes require recognition of RNA with high sequence specificity. Depending on one's perspective, one might consider RNA to be particularly good at conformational rearrangements or particularly prone to folding into and getting trapped in alternative conformations (219, 220).

Nevertheless, the propagation of conformational rearrangements over a large distance may be limited for RNA relative to proteins because of RNA's limited packing and rigidity. This could limit RNA's ability to carry out complex activities that require large-scale and rapid conformational rearrangements, such as allostery and motor function. A possible role of proteins in the spliceosome and ribosome may be to use and control RNA's innate tendency to adopt multiple stable structures. The requirement in splicing and in ribosomal assembly for proteins that are RNA-dependent ATPases suggests that, at least in some cases, conformational changes in RNA are driven by protein enzymes (221, 222).

SELECTIONS OF RNA AND PROTEIN CATALYSTS

In vitro selections and combinatorial approaches are finding applications in a wide range of research (for reviews, see 223–227). Here we contrast the selection processes used to obtain catalytic RNAs with those used to obtain catalytic proteins. Comparison of the resultant catalysts allows us to highlight features of each class of catalyst introduced in previous sections and to introduce new topics.

Thinking up idealized selections to test mechanistic proposals, such as those suggested throughout this review, is easy on paper. But many factors beyond the intrinsic ability of a particular catalyst to catalyze a particular reaction can intercede and affect the number and proficiency of catalysts obtained in a selection. Nevertheless, selection experiments can generate new RNAs and proteins to continue comparative studies. Such studies must be carried out at a detailed molecular level to gain catalytic insights.

Lessons from Comparisons of RNA and Protein Selection Procedures

Bartel & Szostak were able to select, from random pools of RNA, ribozymes that could covalently attach RNA oligonucleotides to their own 5′-ends (19, 19a). The rate enhancement of $\sim 10^9$-fold for this reaction is larger than that typically obtained with catalytic antibodies (228), a surprising finding given

the limitations to RNA catalysis described in the previous sections. Thus, a comparison of the procedures used to select catalytic RNAs and proteins may be informative.

NUMBER OF MOLECULES IN THE POOL Because of the simplicity of manipulating and selecting catalytic RNAs, the pool size can be dramatically larger. Bartel & Szostak began with $\sim 10^{15}$ different RNAs that were $\sim$200 nucleotides in length, whereas the diversity of the mouse immune system is estimated to be $\sim 10^{8}$ (228). Note that 10^{8} is negligible compared to 10^{15}, and also that both of these numbers are negligible compared to 10^{120} and 10^{260}, the total number of RNA and protein sequences, respectively, of length 200. These latter numbers, which are larger than the number of particles in the universe (10^{80}; 228a), illustrate that it is not possible for either the experimentalist or nature to sample all of "sequence space," presenting a fundamental limitation on our exploration of molecular function (see below).

THE TYPE OF SELECTION The RNA selections are based directly on the reaction of interest: In the Bartel & Szostak example, the sequences that can perform the ligation reaction are amplified by use of PCR primers complementary to the small RNA that has been attached. In contrast, selections for catalytic antibodies are indirect, relying on the ability to create a small-molecule transition-state analog that resembles the actual transition state (160, 161, 228); such analogs, though often clever in design, are never perfect transition-state mimics. The two catalytic RNAs obtained by selection for binding to a transition-state analog provide only a small amount of catalysis, 100- to 400-fold, for rotamase and metalation reactions (68, 69). It is not clear if this poor catalysis (relative to other selected RNAs) arises because the selection was not direct, because the transition-state analog was imperfect, or because the rotamase and metalation reactions are inherently more difficult for RNA to catalyze than the phosphoryl transfer reaction.

STARTING FROM A RANDOM POOL VS A DEFINED STRUCTURAL MOTIF The protein selections use a pool of sequences that are variations of a sequence that gives a defined overall 3D fold. As only a very small fraction of random protein sequences are expected to give a well-defined structure, this type of pool may be necessary to ensure that there are enough folded proteins to have a reasonable chance that one or more in the pool has a binding site for the substrate and can catalyze its reaction. On the other hand, the fact that nature has not chosen the antibody motif for enzymes raises the possibility that this motif may be particularly adept for binding a wide array of ligands but not for catalysis.

On the basis of the above discussion, one might consider it a disadvantage to begin with randomized RNA. However, there are several mitigating factors:

1. The pool size for the RNA selections is much larger. Hence, there will be a large number of structured RNAs even if only a small fraction fold. Most large random RNAs do indeed fold, because they are able to form long-range base-pairing interactions (229, 230). The formation of secondary structure presumably leads to additional structure formation.

2. Each long randomized RNA can be considered to consist of a large number of shorter randomized sequences that could fold independently and be catalytic. Indeed, the combinatorial advantage can be even greater because noncontiguous regions could come together to form a catalytic unit. These advantages are mitigated, to an unknown extent, by the increased folding into noncatalytic structures that is possible with a longer RNA.

3. The data on RNA tertiary folds is insufficient to indicate whether a particular fold would be especially suited for catalysis.

4. Finally, the RNA pool used by Bartel & Szostak was not completely random. All of the RNAs in the pool contained the same 5′-terminus. This terminus provided a binding site for the oligonucleotide substrate via simple Watson-Crick base pairing. More generally, the ease of creating a binding site for RNA that is made up of a short, contiguous stretch of RNA solves the first problem of catalysis—localizing the substrate to the catalyst—and is expected to greatly enhance the probability of obtaining catalysts, via selection, with some measurable amount of activity (see also 231).

THE TYPE OF REACTION CATALYZED The difficulty in obtaining catalysts for various reactions is expected to depend on the type of selection. For example, esterase reactions may be one of the easiest types of reactions to catalyze using the transition-state analog binding approach, because there are a plethora of charged, tetrahedral transition-state analogs available. In contrast, phosphoryl transfer catalysts may be difficult to obtain by this approach because there are no good pentavalent transition-state analogs that are stable in solution. Catalysis of phosphoryl transfer may instead be more readily obtained by direct selection using RNA catalysts because these reactions may be particularly sensitive to metal ion catalysis, because RNA may be particularly adept at metal ion catalysis (see above), and because an RNA or DNA substrate is readily bound by an RNA catalyst. Newly developed direct-selection protocols for RNA and DNA should make it straightforward to perform selections for an array of reaction types (232).

OTHER DETERMINING FACTORS IN SELECTIONS Protein selections can give antibodies that bind to only a portion of the transition-state analog and thus provide

no catalysis. RNA selections can produce parasitic RNAs that are unrelated to the desired catalysts (233). Another factor important to the success of a selection is its simplicity of design and execution. For example, the simplicity of selections for DNA catalysts allows a faster generation time per round of selection. This may be in part responsible for the ability to readily select DNA enzymes that can catalyze RNA hydrolysis as efficiently as the hammerhead and other small ribozymes (232, 234). Although we expect that DNA will be more limited in its catalytic proficiency and repertoire than RNA because of the absence of the 2′-hydroxyl group, just as RNA will be more limited than proteins because of RNA's functional group limitations, the ease of performing DNA selections may make up for some of its inadequacies from the practical standpoint of the ability to obtain catalysts from selections.

How Good Can an RNA Catalyst Be and What Is the Repertoire of RNA Catalysis?

We do not know the ultimate ability and repertoire of RNA as a catalyst. Indeed, it is not possible to show that there is a limit to the rate enhancement provided by an RNA catalyst for a particular reaction or that RNA cannot catalyze another type of reaction, because only a vanishingly small portion of sequence space can be searched. More to the point is the question: What is the probability of finding an RNA or protein catalyst that can provide a certain rate enhancement for a particular reaction? We imagine probability distributions like those shown in Figure 9. For a reaction that RNA is well suited to catalyze, such as phosphoryl transfer with an RNA substrate, we expect that short RNAs will be better catalysts than short peptides (Figure 9, arrow "a"). We expect this finding because even short RNA can effectively use duplex formation (secondary structure) to bind RNA substrates, and these RNAs can also bind metal ions; in contrast, protein binding sites typically include residues that come together in the overall tertiary structure from residues that are distant in primary sequence.

However, we suggest that the situation is reversed for larger RNAs and proteins. As described above, creating a precisely positioned three-dimensional structure appears to be more difficult with RNA than with proteins, so a larger RNA would be required to achieve a rate enhancement similar to that for a folded protein (Figure 9, arrow "b"). In addition, even the fastest known RNA enzymes, the *Tetrahymena* group I ribozyme and the RNase P RNA provide rate enhancements that are $\sim 10^3$-fold less than those provided by protein enzymes such as staphylococcus nuclease that catalyze analogous phosphoryl transfer reactions (Figure 9, arrow "c") (26, 28, 46, 235). This difference may also reflect the contribution from specific functional groups of the protein enzymes

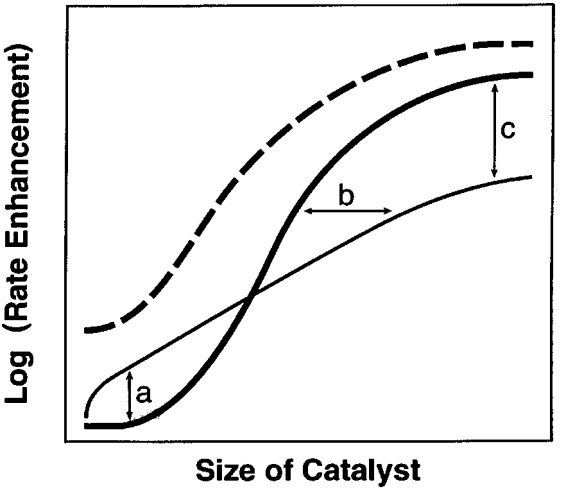


Figure 9 Rate enhancement that can be achieved by RNA (*thin solid line*) or protein (*thick solid and dashed lines*) catalysts of varying size. The lines are purely hypothetical and are presented solely to help conceptualize points presented in the text.

in proton donation or abstraction and in the positioning of the substrate and metal ions in the active site.

The probability distribution will look different for catalysis of different types of reactions. For example, reactions that are particularly prone to chemical catalysis by protein functional groups would be more readily catalyzed by smaller proteins (e.g. Figure 9, dashed line). An example of this may be the catalysis of decarboxylation of oxaloacetate by a small peptide containing multiple Lys residues (236; see also 237). Similarly, a reaction that is not prone to metal ion catalysis would be represented with a lower curve for RNA in a plot analogous to that of Figure 9. Cross-linking of cysteine residues can increase structural integrity and rigidity, possibly allowing greater catalysis from smaller peptides. Nevertheless, smaller peptides are not expected to be capable of forming an extensive binding pocket.

Given this view, we can imagine, as have many others, an early evolutionary scenario with RNA or RNA-like catalysts. We then suggest the following speculative series of events leading ultimately to their replacement with protein catalysts. The first functional peptides bound RNA nonspecifically and aided structural transitions of RNA (220). Although the peptides alone were not good catalysts, once they bound RNA, they then evolved to use reactive functional groups absent in RNA for catalysis. The peptide then replaced the structural

role of RNA by adding residues in a stepwise fashion until the protein was large enough to fold independently and completely replace the RNA.

SUMMARY AND PERSPECTIVE

RNA enzymes have provided the mechanistic enzymologist a new vantage point for viewing an old problem. The details of the analysis presented herein suggest that RNA will not measure up to proteins as catalysts, the same answer apparently arrived at by evolution. But we would like to emphasize not the inadequacies of RNA but rather its benefits: what it has taught us about biological catalysis. By providing a counterpoint to the more familiar protein enzymes, comparisons with RNA enzymes have helped illuminate features of proteins that are important for catalysis. From the standpoint of transition-state theory, the comparisons highlight the ability of proteins to position functional groups within an active site and to manipulate and control the electrostatic nature of the active-site environment. Most fundamentally, the commonality between RNA and protein enzymes in their use of intrinsic binding energy for catalysis suggests that the use of binding interactions away from the site of chemical transformation to facilitate reaction is a fundamental principle of biological catalysis. We suggest that this is so because it inextricably links the two requirements of biological catalysts: exquisite specificity and enormous rate enhancements.

RNA catalysts have also taught us new things. For example, results with the *Tetrahymena* ribozyme suggest that phosphoryl transfer can be catalyzed by electrostatic destabilization of the ground state. More generally, RNA enzymes appear to be particularly tractable to energetic analyses. We anticipate that comparisons of the catalytic behavior of RNA and protein enzymes, obtained both naturally and via selection, will continue to reveal energetic principles of catalysis as well as define qualities of both of these classes of biological macromolecules.

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