A Core Folding Model for Catalysis by the Hammerhead Ribozyme Accounts for Its Extraordinary Sensitivity to Abasic Mutations†

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Received April 17, 1998; Revised Manuscript Received August 4, 1998

ABSTRACT: Introducing abasic nucleotides at each of 13 positions in the conserved core of the hammerhead ribozyme causes a large decrease in the extent of catalysis [Peracchi, A., et al. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93,* 11522]. This extreme sensitivity to structural defects is in contrast to the behavior of protein enzymes and larger ribozymes. Several additional differences in the behavior of the hammerhead relative to that of protein enzymes and larger ribozymes are described herein. The deleterious effects of the abasic mutations are not relieved by lowering the temperature, by increasing the concentration of monovalent or divalent metal ions, or by adding polyamines, in contrast to effects observed with protein enzymes and large RNA enzymes. In addition, the abasic mutations do not significantly weaken substrate binding. These results and previous observations are all accounted for by a "core folding" model in which the stable ground state structure of the hammerhead ribozyme complexed with the substrate is a partially folded state that must undergo an additional folding event to achieve its catalytic conformation. We propose that the peculiar behavior of the hammerhead arises because the limited structural interconnections in a small RNA enzyme do not allow the ground state to stably adopt the catalytic conformation; within the globally folded catalytic conformation, limited structural interconnections may further impair catalysis by hampering the precise alignment of active site functional groups. This behavior represents a basic manifestation of the well-recognized interconnection between folding and catalysis.

The hammerhead (Figure 1) is the smallest known naturally occurring ribozyme (see refs *3*−*5* for review), and the only one for which a complete three-dimensional structure is known to atomic resolution (*6*, *7*). Previously, we began to address the structural basis of hammerhead catalysis through a "subtraction mutagenesis" approach, in which each residue in the conserved core was individually replaced by an abasic nucleotide analogue (Chart 1) (*8*, *9*). Removing individual bases from DNA duplexes induces local, context-dependent rearrangements (*10*−*16*), and removing large amino acid side chains from protein cores (e.g., via alanine scanning mutagenesis) can result in various rearrangements or can leave a cavity within the core (*17*−*19*). Analogously, abasic mutagenesis in the hammerhead core could reveal how this ribozyme responds functionally to the introduction of structural defects and to changes in local packing.

Nearly all of the 13 positions in the hammerhead ribozyme core that were substituted with an abasic residue gave large decreases in the extent of catalysis (*8*) (Figure 1B). In contrast, mutations at only a small subset of "catalytic" residues in proteins typically yield a large decrease in activity (see below). The characterization of the abasic hammerhead variants reported herein was carried out in an effort to understand why so many abasic substitutions in the hammerhead produce such large effects. This characterization, combined with previous observations, suggests that the stable ground state ribozyme−substrate complex is only partially folded and that catalytic interactions are made in a cooperative folding step that precedes the catalytic step. This behavior of the hammerhead ribozyme may arise because this small RNA catalyst lacks the structural redundancy present in protein enzymes with similar molecular masses and in larger RNA enzymes.

MATERIALS AND METHODS

Materials

Oligonucleotides. The wild-type HH16, its variants,[1] and all other RNA oligonucleotides used in this study were prepared by solid-phase synthesis (*20*). The sequences of the HH16 substrates used are shown in Chart 2. The

† This work was supported by NIH Grant GM49243 and by an Established Investigator Award from the American Heart Association to D.H.; A.P. was supported by a Human Frontier Science Program postdoctoral fellowship (LT 867/95).

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[1] E refers generically to the hammerhead ribozyme HH16 and all of its variants. HH16 and its substrate, S, are shown in Figure 1A. Individual abasic variants of HH16 are specified by acronyms analogous to those used for mutant proteins. For example, A9X is a ribozyme where the A at position 9 has been changed to an abasic residue (X). Other abbreviations: HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid.

10.1021/bi980867y CCC: $15.00
Published on Web 10/01/1998

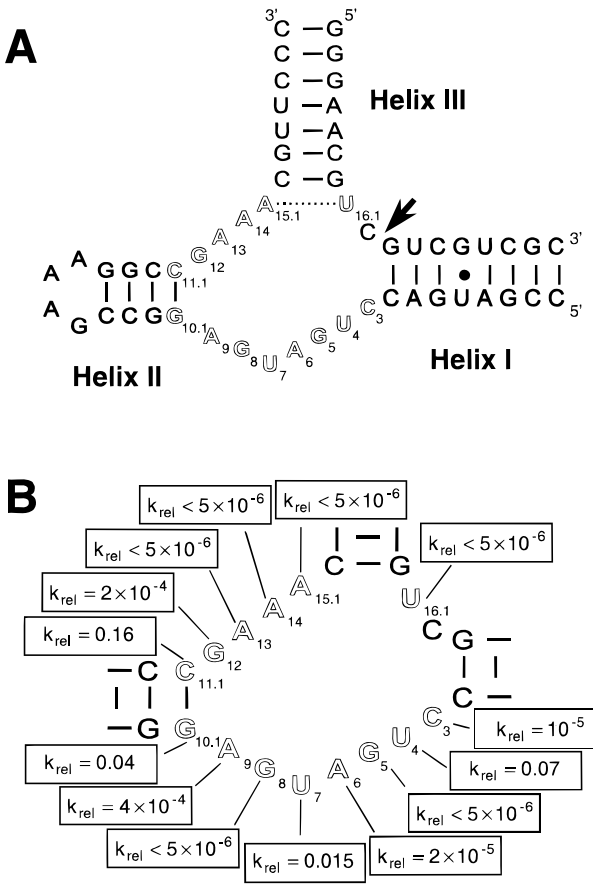


FIGURE 1: Hammerhead ribozyme and abasic variants. (A) Secondary structure of the hammerhead ribozyme, HH16, with bound substrate. The hammerhead consists of three helices and 11 nonhelical residues located in the highly conserved central region. The arrow indicates the position that is cleaved in the substrate strand. Residue numbering follows the standard hammerhead nomenclature (*1*). The positions modified in this study are shown as outlined letters and, except U7, G10.1, and C11.1, are conserved in natural hammerhead isolates (*2*). (B) Reduced activity of abasic HH16 variants. The effects of abasic modifications on the chemical step were presented previously (*8*), except for that for position 16.1, which was investigated in this study. k_{rel} is defined as k_2^{abasic}/k_2^{wt}; k_2^{wt} and k_2^{abasic} are the first-order rate constants for the cleavage of the substrate in the complex with the wild-type and abasic ribozyme, respectively [50 mM Tris (pH 7.5) and 10 mM Mg^{2+} at 25 °C].

incorporation of reduced abasic nucleotides in RNA has been described previously (*9*, *21*).

Ribozymes were purified by anion-exchange HPLC (*20*). Substrates were 5′-^{32}P end labeled with T4 polynucleotide kinase and gel purified. Oligonucleotide concentrations were determined from specific activities for radioactive RNAs and from an assumed residue extinction coefficient at 260 nm of 8500 M^{-1} cm^{-1} for nonradioactive RNAs.

Methods

General Kinetic Methods. Previous studies of the ribozyme used in this study, HH16, have established conditions that allow the cleavage step of the ribozyme−substrate complex to be followed (*22*). All reactions herein were single-turnover, with ribozyme (typically 0.6 μM final concentration) in excess of 5′-^{32}P end-labeled substrate (0.1−1 nM final concentration) and, unless otherwise stated, were carried out in 50 mM buffer and 10 mM Mg^{2+} at 25 °C. The substrate and ribozyme were heated at 95 °C for 2 min to disrupt potential aggregates, then cooled, and equilibrated at the desired temperature before initiating reaction. Unless otherwise specified, reactions were initiated by adding $MgCl_2$ to the premixed ribozyme and substrate. Aliquots were removed at appropriate intervals, and further reaction was quenched; product and substrate bands were separated on 7 M urea/ 20% polyacrylamide gels, and their ratio at each time point was quantitated using a PhosphorImager (Molecular Dynamics). Nonlinear least-squares fits of the data were performed using KaleidaGraph (Synergy Software) or Sigma Plot (Jandel Scientific).

Chart 1

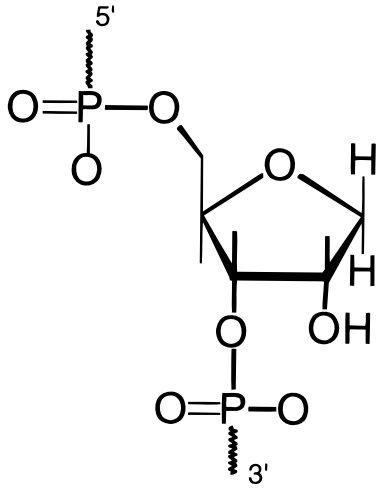
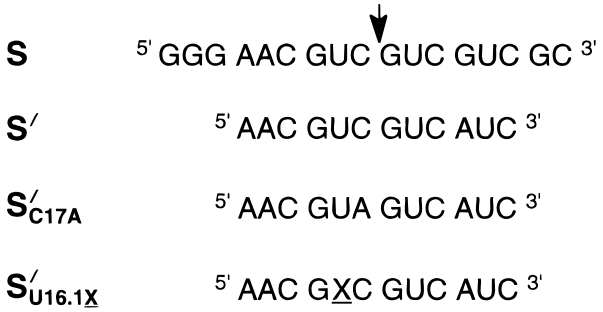


Chart 2



Control reactions in which the concentration of ribozyme was varied indicated that the substrate was completely bound in all cases so that k_2, the first-order rate constant for cleavage of the ribozyme−substrate complex, was followed (see refs *8* and *22* for details). Reactions of the wild-type HH16 and of variants U4X, U7X, G10.1X, and C11.1X were followed to completion, and the reaction time courses fit well to a single-exponential function with an R^2 of >0.99 and end points of 80−90%. For slower variants, the reactions were followed for at least 36 h, and k_2 values were determined from the initial rates, assuming an end point of 90%. The extent of product formation was linear over this time, indicating that there was no significant time-dependent inactivation of the ribozyme.

The buffer used in these reactions was either 50 mM Tris·HCl at pH 7.5 or 50 mM MES·Na at pH 6.5. Use of the pH 7.5 buffer was limited to measurements at $T \leq 40$ °C, because the cleavage step for the wild-type ribozyme is too fast for accurate measurement at higher temperatures; hammerhead catalysis is ~10-fold slower at pH 6.5 than at pH 7.5 (*23*).

Determination of Dissociation Constants. The thermodynamic dissociation constants of the enzyme−substrate complexes (K_d^{ES}) were determined at 35 °C in 50 mM MES·Na (pH 6.5) and 10 mM $MgCl_2$, using a shortened version of the HH16 substrate termed S′ (Chart 2).[2] The elevated temperature and the shortened substrate were employed to ensure equilibration prior to cleavage in single-turnover kinetic experiments.

For the wild-type HH16, as well as for the abasic variants retaining a significant activity (variants U4X, U7X, A9X, G10.1X, C11.1X, and G12X), K_d^{ES} was obtained from kinetic experiments in which the extent of cleavage of S′ (∼0.2 nM) was measured as a function of increasing concentrations of E (3−5000 nM). In these experiments, a solution containing labeled S′ and another solution containing E were heated separately at 95 °C for 2 min and then cooled to room temperature. $MgCl_2$ was added to each solution, and the tubes were equilibrated at 35 °C for 10 min before initiating the reaction by mixing the ribozyme and the substrate solutions. The reaction is described by eq 1.

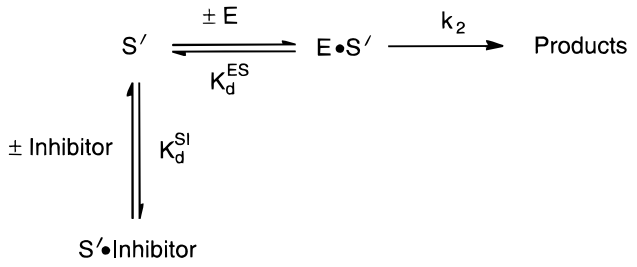
$$E + S' \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}S' \xrightarrow{k_2} \text{products} \qquad (1)$$

For the wild type and abasic hammerhead variants, the time courses fit well to a single-exponential function ($R^2 > 0.99$), with no lags or multiple phases. Furthermore, the reaction profiles at a given E concentration were identical to those of controls in which the E and S′ were preincubated together 10 min before initiating the reaction by addition of Mg^{2+}. These observations are consistent with equilibration of the substrate between bound and unbound forms being fast compared to cleavage ($k_{-1} \gg k_2$). Under these conditions, eq 2 describes the dependence of the observed cleavage rate constant on E concentration and allows the dissociation constant for the ribozyme−substrate complex, K_d^{ES}, to be determined.

$$k_{obs} = \frac{k_2[E]}{K_d^{ES} + [E]} \qquad (2)$$

The following experiments with wild-type HH16 provided further evidence that equilibration of the substrate is fast ($k_{-1} \gg k_2$) so that K_d^{ES} can be obtained from the dependence of k_{obs} on the E concentration (eq 2); this is also expected to hold for the abasic ribozymes, as the abasic variants exhibit reduced values of k_2. (i) Results from dilution-chase experiments (*22*, *24*) with wild-type HH16 well above the apparent K_d^{ES} ([E] = 800 nM vs K_d^{ES} = 220 nM) suggested that S′ dissociates immediately upon dilution, without further reaction of the E·S′ complex. (ii) Decreasing the pH from 6.5 to 5.2 had no effect on the measured K_d^{ES} within error (K_d^{ES} = 220 ± 80 and 150 ± 50 nM at pH 6.5 and 5.2, respectively), despite the 50-fold decrease in k_2 from 0.4 to 0.008 min^{-1}.

Scheme 1



The value of K_d^{ES} for dissociation of $S'_{U16.1X}$ (Chart 2) from the wild-type ribozyme was obtained by following inhibition of the ribozyme reaction with 5′-end-labeled S′ by added $S'_{U16.1X}$ under conditions in which the reaction rate is linearly dependent on the concentration of free ribozyme (10 nM HH16, 0.2 nM S′, and 50−10000 nM $S'_{U16.1X}$). For ribozymes that are essentially inactive (variants C3X, G5X, A6X, G8X, A13X, A14X, and A15.1X), K_d^{ES} was determined in inhibition experiments described in the next section.

Substrate Inhibition for Determination of Dissociation Constants. The "substrate inhibition" method allows determination of the stability of RNA·RNA complexes directly under reaction conditions (*25*). In contrast to standard competitive inhibition, in which an inhibitor blocks reaction by binding to the enzyme, in substrate inhibition the inhibitor blocks reaction by complexing with the substrate (Scheme 1).

Substrate inhibition experiments were used to determine (i) the affinity of S′ for inactive or barely active ribozyme variants and (ii) the stability of duplexes formed by either S′ or S'_{C17A} (Chart 2) with short complementary RNA strands (see Figure 8 below). Reactions were carried out in 50 mM MES·Na and 10 mM Mg^{2+} at 35 °C, matching the conditions used in the other K_d^{ES} determinations. Experiments were carried out as follows. A solution containing the labeled substrate (S′ or S'_{C17A}) and another solution containing the wild-type ribozyme (HH16) and the inhibitor (either an inactive ribozyme or a short RNA strand complementary to the substrate) were heated separately at 95 °C for 2 min to disrupt potential aggregates and then cooled to room temperature. $MgCl_2$ was added to each solution, and the solutions were equilibrated at 35 °C for 10 min before initiating the reaction by mixing the ribozyme−inhibitor and substrate solutions (Scheme 1). The final concentrations were 20 nM for HH16 and ∼0.2 nM for the substrate, with varying concentrations of inhibitor. The decrease in the observed cleavage rate constant (k_{obs}) as a function of the inhibitor concentration was fit to eq 3

$$k_{obs} = \frac{k^{\circ}_{obs}}{1 + [\text{inhibitor}]/K_i} \qquad (3)$$

in which k°_{obs} is the rate constant observed in the absence of inhibitor and K_i is the inhibition constant (*25*). The inhibitor concentrations used in these experiments ranged from at least 4-fold above to 4-fold below K_i. The HH16 concentration was more than 10-fold lower than K_d^{ES} (K_d^{ES} = 220 and 650 nM for S′ and for S'_{C17A}, respectively), ensuring

[2] In addition to being shortened, S′ also contains an A residue three nucleotides from its 3′-end, corresponding to a position at which S, the normal substrate, has a G residue (Chart 2). As a result, the HH16·S′ complex contains a standard Watson−Crick A·U pair in helix I in place of the G·U wobble pair of the HH16·S′ complex (Figure 1). This G to A modification prevents S′ from binding predominantly in an alternative, unproductive mode (unpublished results). The modification does not have any appreciable effect on the rate of catalysis; i.e., with a saturating ribozyme level, cleavage of S′ and cleavage of S occur at the same rate.

that essentially all of the substrate present is not complexed with ribozyme. Under these conditions, the observed inhibition constant is expected to be equivalent to the dissociation constant for complex formation between the inhibitor and S′ (i.e., $K_i = K_d^{SI}$, Scheme 1) (*25*).

For K_i to reflect K_d^{SI} (Scheme 1), it is also necessary that the complex between the substrate and the inhibitor equilibrate fast relative to cleavage and that the inhibitor not aggregate (see ref *25* for a more complete discussion). The following results suggest that these criteria also hold. (i) In all cases, disappearance of the substrate followed single-exponential kinetics. (ii) Decreasing the ribozyme concentration to 4 nM or lowering the pH to 5.2 reduced the observed cleavage rate (by ~5 and ~50-fold, respectively) but did not affect K_i, suggesting that dissociation of the inhibitor−substrate complex was not rate-limiting under these reaction conditions. (iii) The plots of k_{obs} as a function of the inhibitor concentration consistently fit well to the simple inhibition curve of eq 3 ($R^2 > 0.99$), providing no indication of aggregation of the inhibitor or other kinetic complexities.

BACKGROUND

Extraordinary Sensitivity of the Hammerhead Ribozyme to Mutations. We previously reported that abasic mutations substantially decrease the rate of cleavage by the hammerhead ribozyme (Figure 1B) (*8*). For 10 of the 14 positions within the conserved core, removal of the base decreases the cleavage rate by $>10^3$-fold, corresponding to an effect of >4 kcal/mol at 25 °C. In six of these cases, the decrease of ~10^6-fold reduces cleavage essentially to the background rate.

In contrast to the profound sensitivity of the hammerhead to mutation, only a small subset of residues in protein enzymes yield large decreases in the extent of catalysis upon mutation. The following series of examples illustrates this difference.

RNase T1 catalyzes a phosphodiester cleavage reaction identical to that of the hammerhead and has a molecular mass similar to that of the ribozyme (~11 vs ~10 kDa for a "minimal" hammerhead structure that includes the central core and three base pairs in each helix). Mutations at 19 different positions located at or near the active site have been characterized for this enzyme (*26*−*38*), yet mutations at only two positions decreased k_{cat} with GpC by more than 100-fold (*38*); these positions correspond to the active site His40 and His92 that have been proposed to act in general acid and base catalysis (*27*, *38*).

Eighteen conserved residues of a cytosolic glutathione *S*-transferase (17 kDa) were individually mutated in a series of studies (*39*−*41*). Mutations at only two positions, including Tyr9, which is thought to be involved in substrate activation, produced decreases in k_{cat} of >100-fold.

The α-subunit of tryptophan synthase (29 kDa) was subjected to random mutagenesis, and 66 single-site mutants at 49 different positions were isolated. Mutations at only two of these positions were found to decrease activity by >100-fold (*42*). These two positions correspond to the active site residues Glu49 and Asp60 that are thought to be involved in general acid and base catalysis.

Mutations at 30 positions at or near the active site of the 29 kDa carbonic anhydrase II have been characterized (*43*−*53*), but only mutations of five residues decreased k_{cat}/K_M by more than 100-fold. These include the three His residues involved in coordination of the catalytic zinc (*50*).

In a study on staphylokinase (18 kDa), 18 mutant enzymes were characterized in which clusters of two to three charged residues were changed to alanine (*54*). For 15 of these mutants, k_{cat} toward the substrate plasminogen was within 5-fold of the wild-type value, and none showed a decrease in activity larger than 40-fold (*54*).

The difference between the behavior of the hammerhead and that of protein enzymes does not appear to be accounted for simply by the drastic nature of the abasic mutation. In a previous hammerhead study, 11 residues in the conserved core were individually mutated to each of the other three natural nucleotides; 23 of the 33 mutants had activity decreases of >100-fold (*55*). In addition, the conservative substitution of individual nonhelical guanines in the core, G5, G8, and G12, by 2-aminopurine each decreased activity by $>10^3$-fold (*56*). In contrast, individually mutating each of the seven Trp residues in carbonic anhydrase II to smaller residues (either Phe, Cys, or, in one case, Gly) decreased k_{cat} by 5-fold at most (*48*, *51*).

In summary, mutagenesis of the hammerhead core reveals a much larger fraction of "essential" residues and groups than is typically found in mutagenesis studies of protein enzymes. Some of this difference could arise because several hammerhead residues may be required for coordinating one or more catalytic metal ions (*57*−*65*). However, it would appear unlikely that all bases and functional groups yielding large effects upon mutation are directly involved in catalysis, or coordinate a metal ion that is directly involved in catalysis. Instead, many of the mutations could impair function indirectly, by affecting the structure of the core. The following section describes a model that describes how these structural effects can be expressed as defects in catalysis and why this behavior is distinct from that of protein enzymes and larger ribozymes. This model is supported by previous results, and further support is provided by results with the abasic ribozymes that are described in the Results and Discussion.

A "Core Folding" Model for Hammerhead Catalysis. There is substantial evidence for a conformational transition prior to cleavage of bound substrate by the hammerhead ribozyme. The initial X-ray structures of hammerhead−oligonucleotide complexes revealed that the scissile phosphoryl group was not properly aligned for an in-line nucleophilic attack by the 2′-hydroxyl (*6*, *7*), and several residues and functional groups that are critical for catalysis make no interactions or limited interactions in these ground state structures (reviewed in ref *4*; see also ref *66*) (Figure 2). Furthermore, results from rescue experiments of C3X by addition of exogenous bases (*8*; A. Peracchi, J. Matulic-Adamic, S. Wang, L. Beigelman, and D. Herschlag, in press in *RNA*) suggest that C3 forms transition state interactions with its base-pairing face that are not present in the ground state structure. In addition, a metal binding site identified near G5 by structural, kinetic, and spectroscopic analysis has been shown to be inhibitory, suggesting the occurrence of a conformational transition involving or near G5 (*67*; A. Feig and O. Uhlenbeck, unpublished results) (Figure 2). Finally, a distinct metal ion was identified in the X-ray structure ~20 Å from the cleavage site phosphodiester, with no obvious

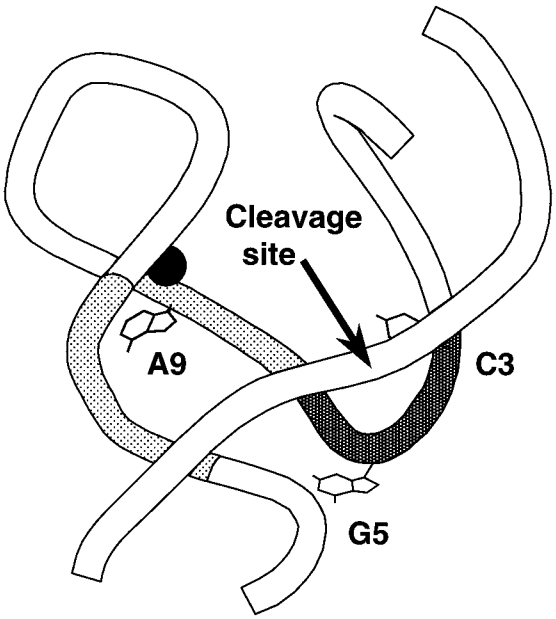


FIGURE 2: Schematic representation of the three-dimensional structure of the hammerhead ribozyme (based on ref *6*). Domain I of the core is shown by the dark stippled region and domain II by the light stippled region. Some of the residues referred to in the text are shown explicitly. The black sphere near residue A9 represents a metal ion identified crystallographically (*6*) and probed in functional studies (*65*).

structural connection to the cleavage site (Figure 2) (*6*). This metal ion nevertheless appears to be critical for catalysis, and an analysis of functional data suggested that this metal ion adopts at least one additional ligand in the catalytic conformation (*65*). Thus, the X-ray structures (*6*, *7*) suggest that the core in the ground state ribozyme−substrate complex is structured to some degree, but the above observations suggest that additional connections are made in the catalytic conformation.

The effect of the abasic mutations in the hammerhead core, if energetically additive, would yield an overall effect of $>10^{54}$-fold (Figure 1B), vastly greater than the observed rate enhancement of 10^6-fold (*68*). Such a cooperative effect is a hallmark of macromolecular folding. This observation, combined with those described above, led to a working model in which the ribozyme undergoes a folding transition to adopt its catalytic conformation (Figure 3). This model can account for the large, cooperative effect of mutations within the core without invoking direct involvement of each residue in catalysis; i.e., each residue contributes to stabilization of the active conformation of the hammerhead, and because these interactions are not present in the starting ground state structure, each residue contributes substantially to catalysis. The widespread large effects of the abasic substitutions and the other observations cited above are not predicted from a simple model in which the overall catalytic structure is stably formed for both the wild-type and modified ribozymes. Further, a hybrid model in which an additional folding step is required for the modified ribozymes but not for the wild-type ribozyme is not supported by the divergence between the groups involved in ground state interactions in the X-ray crystal structure of the wild-type ribozyme and the groups important for catalytic function, as described above. Additional properties of the abasic hammerhead variants are described in the Results and Discussion, each of which is not easily reconciled with either alternative model and all of which provide support for the core folding model of Figure 3.

RESULTS AND DISCUSSION

The Hammerhead Core Has the Same Response to Mutations as a Protein Enzyme That Is Unfolded: Absence of Rescue at Lower Temperatures. Folded protein enzymes can be substantially destabilized by mutations at many positions,[3] although, as pointed out above, the subset of residues giving large effects on catalysis is much smaller. For example, the Pro39Ala mutation in RNase T1 decreases the stability of the folded enzyme by 4.3 kcal/mol, whereas catalytic activity of the folded mutant protein is not decreased (*33*). Forty-one of 66 random mutants of tryptophan synthase α from *Escherichia coli* were found to be substantially less stable than the wild type to thermal inactivation, but only four of these exhibited a decrease in specific activity of more than 2-fold at 37 °C (*42*, *75*).

Thus, if a protein enzyme were assayed at a temperature above its unfolding temperature, large deleterious effects from many mutations would be expected. This is because the protein would have to fold prior to catalyzing its reaction so that mutations that decrease in stability would decrease in catalysis, analogous to the proposal for the hammerhead in the core folding model (Figure 3). However, the deleterious effects of most of the protein mutations would be expected to be rescued[4] by lowering the temperature below the unfolding transition for the wild type and mutants, as depicted in Figure 4. Analogously, if the wild-type hammerhead were folded and the mutants unfolded under normal assay conditions, then lowering the temperature would be expected to rescue the deleterious effect and increasing the temperature would be expected to exacerbate it. In contrast, no such rescue or exacerbation is predicted for the hammerhead ribozyme by the "core folding" model of Figure 3. This is because, according to the model, the stable ground state complex remains predominantly unfolded so that the folding transition is required at all temperatures for both the wild-type and mutant ribozymes.

Figure 5 shows the effect of temperature on the activity of abasic hammerhead variants relative to that of the wild type. The abasic variants are greatly compromised in catalytic efficacy even at 0 °C, the lowest temperature tested. In no case is there substantial rescue, consistent with the prediction from the core folding model. Nevertheless, the possibility that the lack of rescue at low temperatures results

[3] Staphylococcal nuclease (14 kDa) has been used extensively in protein folding studies. Of the 345 characterized mutants of this enzyme, 56% show a decrease in stability of >1 kcal/mol (*69*−*72*). Eighty-three of these mutations involved replacement of a large amino acid residue (Tyr, Phe, Met, Val, Ile, or Leu) with either Ala or Gly. These mutations, which are the most similar in extent to abasic replacements in RNA, destabilized staphylococcal nuclease by an average of 3.3 kcal/mol (*69*). Even larger effects have been observed with "subtraction mutagenesis" involving Trp residues. For example, the Trp14Ala mutation in the Arc repressor reduces the protein stability by 4 kcal/mol (*73*). The Trp28Ala mutation in thioredoxin also destabilizes the folded state by ∼4 kcal/mol (estimated from data in ref *74*), without significantly impairing function.

[4] The term "rescue" is used to denote the increase of activity of a mutant relative to the wild type. Differential activation can elucidate functional differences between the two catalysts. Accordingly, the kinetic data on the abasic mutants are reported here mainly as k_{rel}.

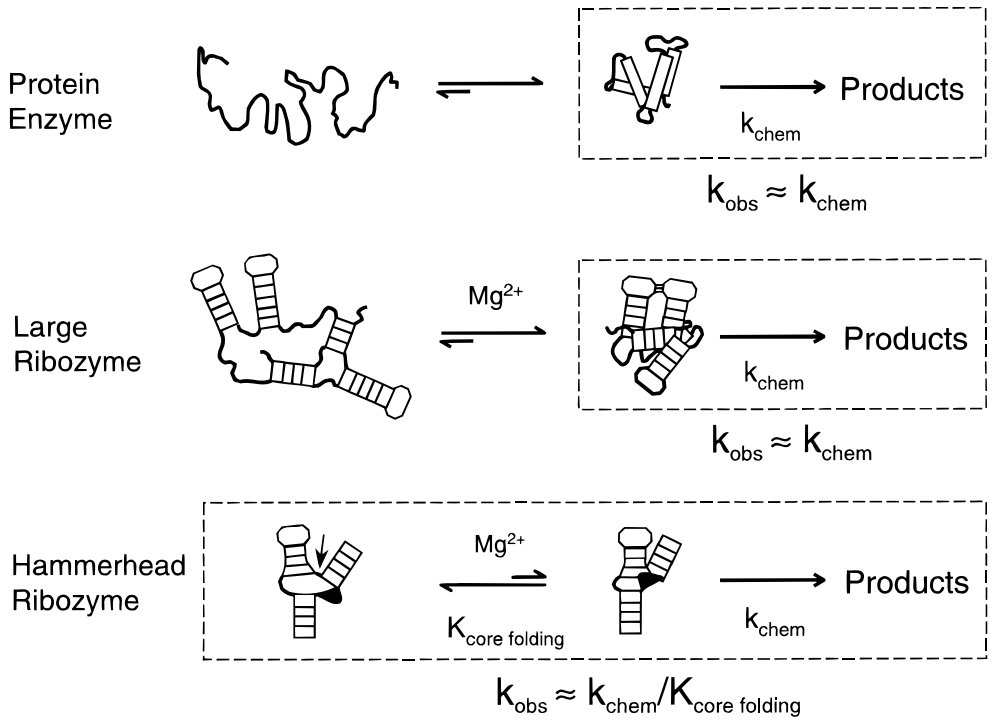


FIGURE 3: Core folding model. Protein enzymes (top) and large ribozymes (middle) show a favorable free energy for folding under physiological conditions. Thus, in both protein enzymes and large ribozymes, the catalytic process, depicted within the dashed boxes, begins with a fully folded catalyst. In contrast, according to this model, the most stable conformation of the hammerhead ribozyme (bottom) is only partially folded. This ground state is referred to as "unfolded" for simplicity in parts of the text. A conformational change that leads to the formation of a transient, more compact structure would be required for hammerhead catalysis. This "folding step" could entail domain I in the ribozyme core (in black) docking onto domain II (*65*).

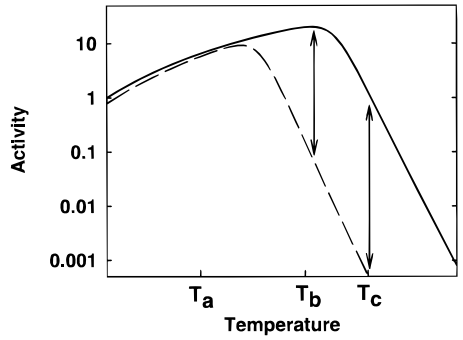


FIGURE 4: Hypothetical temperature dependence for the activity of a wild-type (—) and of a mutant (— —) protein enzyme. The two enzymes exhibit temperature profiles with the same overall shape, but thermal denaturation occurs at lower temperatures for the less stable mutant. Thus, at physiological temperature (T_a), there is no significant difference in activity, but the difference in activity is large at a more elevated temperature (T_b) at which the mutant but not the wild-type enzyme unfolds and at higher temperatures (T_c) that result in predominant unfolding of both enzymes.

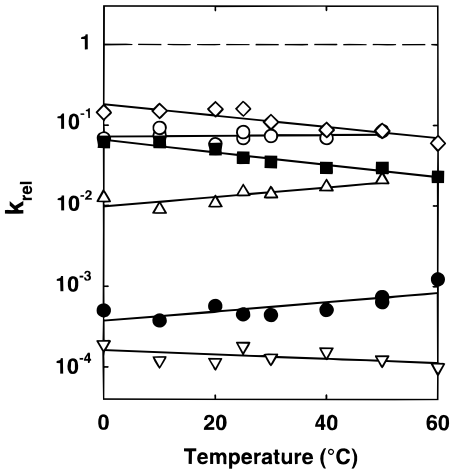


FIGURE 5: Temperature dependence of the cleavage reaction for the wild-type HH16 and for six partially active abasic hammerhead variants. Single-turnover reactions were carried out in the presence of 10 mM Mg^{2+}, in 50 mM HEPES·Na (pH 7.5, 0 °C), 50 mM Tris·HCl (pH 7.5, 10−40 °C), or 50 mM MES·Na (pH 6.5, 40−60 °C), as described in Materials and Methods. The pH of each buffer was adjusted at the final desired temperature. At each temperature, k_{rel} was calculated as k_2^{abasic}/k_2^{wt}: U4X (○), U7X (△), A9X (●), G10.1X (■), C11.1X (◇), and G12X (▽). Solid lines through each set of data points are shown to illustrate the trend in the data. By definition, k_{rel} for the wild type is equal to 1 at each temperature (dashed horizontal line). k_2^{wt} increases by approximately 1000-fold between 0 and 60 °C.

from an absence of a significant temperature dependence for folding of the abasic variants cannot be eliminated.

This behavior of the hammerhead is also distinct from that of larger RNA enzymes. For example, a G212C mutant of the *Tetrahymena* group I ribozyme splices ~1000-fold more slowly than the wild type at 50 °C, but this difference reduces to only ~20-fold at 30 °C (*76*; see refs *77*−*79* for additional examples).[5] Further support for a distinction between the hammerhead and large RNA enzymes comes from the

[5] It is not known what catalytic step was being monitored in the mutational studies on large ribozymes and many of the protein enzymes discussed in the text. Indeed, turnover for protein and RNA enzymes is often limited by steps other than the chemical step (*22*, *103*, *112*). Mutations giving a small or moderate decrease in the rate of the chemical step will not appreciably change the turnover rate if nonchemical steps are predominantly rate-limiting, so the observed effects can underestimate the impact of a given mutation on chemical catalysis.

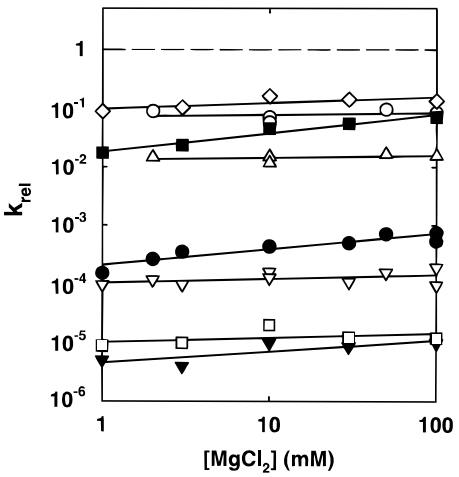


FIGURE 6: Increasing Mg^{2+} concentration does not efficiently rescue a series of partially active hammerhead variants. Single-turnover reactions of HH16 and the abasic variants were carried out in 50 mM Tris·HCl (pH 7.5, 25 °C) in the presence of $MgCl_2$ at increasing concentrations. At each Mg^{2+} concentration, k_{rel} was calculated as k_2^{abasic}/k_2^{wt}: C3X (▼), U4X (○), A6X (□), U7X (Δ), A9X (●), G10.1X (■), C11.1X (◇), and G12X (▽). Straight lines through each set of data points are shown to illustrate the trend in the data. By definition, k_{rel} for the wild-type level is equal to 1 at each Mg^{2+} concentration (dashed horizontal line). k_2^{wt} increased by approximately 30-fold between 1 and 100 mM Mg^{2+}.

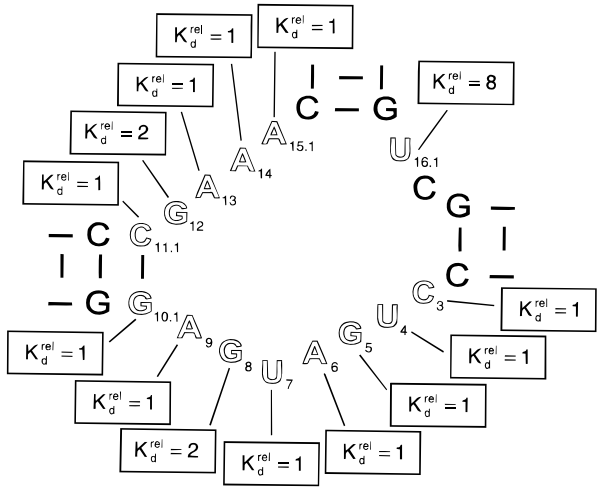


FIGURE 7: Affinity of abasic variants for the hammerhead substrate. K_d^{rel} is defined as $K_d^{ESabasic}/K_d^{ESwt}$; K_d^{ES} is the thermodynamic dissociation constant of the E·S′ complex. Values of K_d^{ES} were measured in 50 mM MES·Na and 10 mM $MgCl_2$ (pH 6.5, 35 °C) as described in Materials and Methods. Under these conditions, K_d^{ESwt} is 220 ± 80 nM.

magnitude of the effect of core mutations. As noted above, 22 out of 33 mutations in the hammerhead core decreased activity >100-fold (*55*); in contrast, mutations at only 10 out of 55 positions in the core of the *Tetrahymena* group I ribozyme decreased the extent of self-splicing by more than 100-fold (*80*).[5]

The Response of the Hammerhead to Mutations Is Different Than the Response of Larger RNA Enzymes: Absence of Mg^{2+} Rescue. The effects of mutations in the larger ribozymes have been shown, in many cases, to be rescued by increasing the concentration of Mg^{2+} (*76*, *77*, *81*−*87*). In some instances, increasing the concentration of monovalent cations or addition of a polyamine can also provide rescue (*78*, *79*, *84*, *87*, *88*). This rescue presumably arises from the creation of metal binding sites, especially for divalent metal ions, in a folded RNA, so that added Mg^{2+} stabilizes the folded RNA relative to unfolded structures (*89*−*94*). Divalent and monovalent cations and polyamines can also aid in RNA folding if there is an increase in the packing density of the charged phosphodiester backbone upon folding and if the spacing of the negative charges in the folded structure matches that of the positive charges in the polyamine.

We therefore tested the ability of cations to rescue the deleterious effect of the abasic mutations in the hammerhead. Increasing the Mg^{2+} concentration to 100 mM did not provide substantial rescue for any of the abasic variants (Figure 6).[6] Similarly, no rescue was observed with high concentrations of monovalent cations (1 M NaCl) or with polyamines (2 mM spermine or spermidine) (data not shown).

The absence of rescue by Mg^{2+} and other cations is in agreement with the core folding model of Figure 3. Mg^{2+} binding sites that are created in the folded, active conformation would always be unoccupied in the ground states for both the wild-type and mutant ribozymes, so changing the concentration of Mg^{2+} would not differentially affect the wild-type and mutant hammerheads. Analogously, if the ground state structure is partially folded in all cases, charge screening effects of cations would aid the wild type and abasic variants similarly, as observed, rather than providing a differential stabilization.

Despite Their Large Effects on the Cleavage Step, Abasic Substitutions Have Little Effect on Substrate Binding. Introduction of an abasic residue at any position within the ribozyme core had little or no effect on the equilibrium constant for dissociation of the ribozyme−substrate complex (K_d^{ES}, Figure 7). Previous mutational work had shown that many mutations within the core did not significantly affect the apparent substrate affinity, although it was not always established that the observed K_m or $K_{1/2}$ value reflected an actual equilibrium dissociation constant (*4*). The results depicted in Figure 7 strengthen these previous observations, as removal of a base eliminates all the interactions involving that base and equilibrium dissociation constants were determined for each residue in the conserved core. With the exception of U16.1,[7] mutations in the conserved core produce no significant effects on the extent of substrate binding, consistent with the minimal connections observed between the core and substrate in crystal structures of ribozyme−substrate analogue complexes (*6*, *7*).

[6] The 3−5-fold increase in k_{rel} with increasing Mg^{2+} concentration observed with variants A9X and G10.1X (Figure 5) may reflect effects from a specific metal ion binding site (*6*, *7*, *65*).

[7] The modest decrease in affinity of 8-fold for $S'_{U16.1X}$ (Figure 7) is consistent with the observation of interactions of U16.1 in the ribozyme crystal structure. This nucleotide appears to form three hydrogen bonds to residues on the ribozyme (*6*, *7*). It should also be recognized that some of the observed affinity difference could arise because the affinity of $S'_{U16.1X}$ for the wile-type ribozyme was obtained by varying the concentration of this substrate in excess of the ribozyme, whereas the affinity of the substrate for the variant ribozymes was obtained by varying the concentration of the ribozyme in excess of the substrate.

It is paradoxical that nearly all of the residues in the hammerhead core are required for catalysis, whereas the apparent interactions of these residues with the substrate are minimal. Interactions beyond base pairing with the substrate must exist and presumably would be extensive to allow catalysis (see also refs *66* and *68*). Although the energy from binding interactions is often not "expressed" in the ground state but is rather expressed in the transition state to give transition state stabilization and catalysis (*95*), it is surprising that only a single core residue produces an effect on binding.

This paradox is readily accounted for by the core folding model of Figure 3. According to this model, interactions responsible for stabilizing the transition state and performing catalysis are not present in the ground state structure, but are formed following the conformational transition. This is analogous to the model suggested to explain the results of mutagenesis in a flexible loop of tyrosyl-tRNA synthetase (*96*, *97*). Thr40 and His45 in the loop do not interact directly or contribute energetically to binding of the tyrosyl-AMP substrate, but have a profound effect on the chemical step, suggesting that the mobile loop is positioned in the transition state to allow interactions with these residues.

The Effect of Abasic Mutations in the Hammerhead Core Is Greater Than in an RNA Duplex. To provide a context for evaluating the magnitude of the effects of abasic mutations, individual abasic mutations were introduced in short oligoribonucleotides, and the impact of these mutations on the duplex stability was determined. The duplexes investigated and observed effects are summarized in Figure 8. Abasic substitution of a single base decreased the duplex stabilities 400−4000-fold[8] (Figure 8). The impact of many of the abasic mutations on hammerhead catalysis is greater than this (Figure 1B). This substantially larger effect is paradoxical if only the ground state structure is considered, as the interactions formed by many of the bases in the hammerhead core do not appear to be as extensive as the hydrogen bonding and stacking interactions within a duplex (*6*, *7*). However, the larger effects of abasics in the hammerhead core are consistent with more extensive interactions of the bases within the core in the folded, active conformation (Figure 3). It is also reasonable that some of the additional sensitivity of the hammerhead to abasic mutagenesis arises from more stringent geometrical constraints for transition state stabilization relative to duplex formation (see Implications and Conclusions).

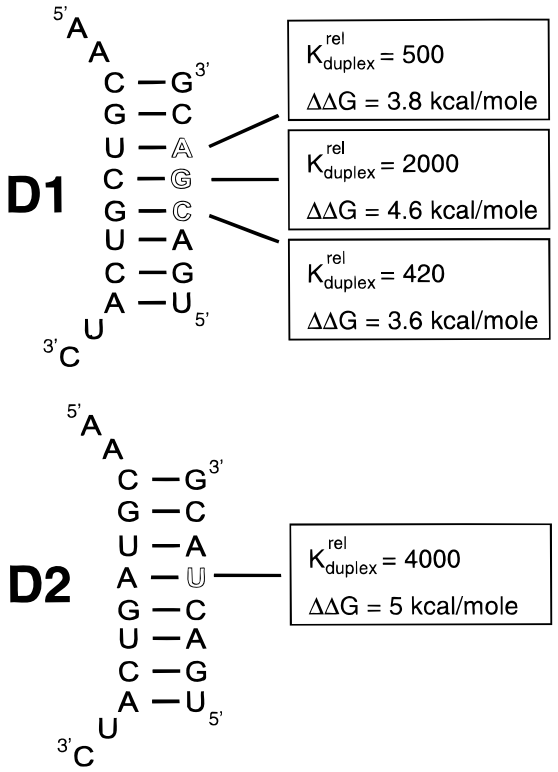


FIGURE 8: Effect of abasic residues on the stability of model RNA duplexes. Each duplex was formed between a short HH16 substrate (S′ for duplex D1 and S'_{C17A} for duplex D2) and a complementary trap oligonucleotide. K_{duplex} values were 5 ± 2 and 2 ± 1 nM for D1 and D2, respectively, and were determined as described in Materials and Methods [50 mM MES·Na and 10 mM $MgCl_2$ (pH 6.5, 35 °C)]. The dissociation constants of the duplexes containing abasic substitutions were determined and used to calculate the change in duplex stability ($\Delta\Delta G$) according to the formula $\Delta\Delta G = RT \ln(K^{rel}_{duplex})$, with K^{rel}_{duplex} defined as $K^{abasic}_{duplex}/K_{duplex}$.

IMPLICATIONS AND CONCLUSIONS

The characterization of abasic hammerhead ribozymes herein combined with previous observations suggests a model in which this small catalytic RNA motif is only partially folded in its ground state with bound substrate and must undergo a folding transition prior to chemical cleavage, as depicted schematically in Figure 3. This behavior is strikingly different from the behavior of protein enzymes and larger ribozymes. The structural differences that may underlie the distinct functional behavior of the hammerhead are discussed below. Analysis of these differences illuminates some fundamental features of biological catalysis.

Structural Redundancy in Proteins versus RNA. The structural problem of the hammerhead apparently does not arise simply from the small size of this ribozyme; even a protein such as RNase T1, which is similar in molecular mass to the hammerhead and carries out the same reaction, is much less sensitive to mutation (*26*−*38*) and much more catalytically proficient. At 25 °C, k_{cat} is ∼2 × 10^4 and ∼1 min^{-1} for RNAse T1 and for the hammerhead, respectively (*29*, *68*).[5]

We suggest that the difference between the behavior of the hammerhead RNA enzyme and protein enzymes arises largely from a scarcity of structural interconnections within the folded RNA structure relative to proteins. The diversity in size, shape, and polarity of the amino acid side chains allows proteins to fold via formation of a closely packed hydrophobic core and networks of hydrogen bonding interactions. The resulting extensive structural interconnections, in turn, prevent an overall collapse of the structure upon removal of individual side chains (*17*−*19*, *104*). This can be described as "structural redundancy". In contrast, RNA side chains are limited in number and diversity; all four bases are planar aromatic groups decorated with hydrogen bond donors and acceptors. This limited diversity combined with

[8] This corresponds to 3.5−5 kcal/mol at 35 °C (Figure 8). In double-stranded DNA, abasic nucleotide analogues were found to decrease the duplex stability by 4−6 kcal/mol at the same temperature (*98*−*100*). The stabilization provided by a base pair is typically larger in the context of an RNA duplex than in a DNA duplex (*101*, *102*) so that the smaller destabilization produced by some abasic residues in RNA is surprising.

the charge and greater number of degrees of freedom in the RNA backbone relative to the protein backbone may hinder close packing within a core and thereby limit the structural interconnections (*105*−*107*).

Thus, more extensive structural interconnections are anticipated for a folded protein than for a folded RNA with a similar molecular mass. In particular, the small hammerhead motif may lack the interconnections needed to stabilize a fully folded, catalytic structure, so any structurally destabilizing mutation would translate into a functional defect. These structural limits are expected to be general for small RNA catalysts. Consistent with this expectation, the hairpin ribozyme, which has a molecular mass of ∼18 kDa, shows a sensitivity to mutations approaching that of the hammerhead. Upon individual mutation of 24 residues of a cis-cleaving hairpin construct to each of the other three natural nucleotides, 36 of the resulting 72 mutants exhibited activity decreases of >100-fold (*108*, *109*). Similarly, in a trans-cleaving hairpin construct, 17 out of 36 single-site mutants had k_{cat} decreases of >100-fold (*110*). In addition, five of ten variants with abasic nucleotides introduced in the hairpin ribozyme core showed decreases in activity of >100-fold (*111*).

How Does RNA Deal with Its Limited Structural Interconnections? As described above, the behavior of the hammerhead in response to mutations appears to differ from that of larger RNA enzymes. For example, deleterious mutations in large ribozymes are often rescued by increased metal ion concentrations or decreased temperature, contrary to what is observed for the hammerhead. In addition, the larger ribozymes can achieve rate enhancements well above the increase in rate provided by the hammerhead (*68*, *112*−*115*).[9] These data, together with the results of structure probing and of crystallographic investigations (*93*, *116*−*118*), suggest that the larger RNA enzymes can adopt stable folded structures.

Phylogenetic analysis, especially of group I self-splicing introns, suggests an approach that these RNAs have taken to solve their folding problem. The group I introns contain a universally conserved core as well as additional domains that are conserved only among subclasses of these introns (*119*, *120*). Three-dimensional modeling and structural mapping studies suggest that these semiconserved regions lie on the periphery of the conserved "core" (*116*−*120*), and structural studies combined with thermodynamic and functional analyses suggest that these peripheral regions are used to stabilize the correct folded state of the core (*88*, *116*, *117*, *119*) and to ensure cooperative folding (D. S. Knitt, E. Doherty, M. Engelhardt, J. A. Doudna, and D. Herschlag, unpublished results).

These peripheral structured regions in large ribozymes may create interconnections, circumventing the inherent structural limits of RNA. For example, the *Tetrahymena* group I intron is made up of 414 residues (molecular mass of ∼130000 kDa), more than 10 times larger than the hammerhead core. Recent structural and functional analysis of a *Tetrahymena* group I construct missing the P5abc peripheral domain (*88*) suggests that the deletion construct behaves more like the hammerhead, requiring a new folding transition prior to catalysis (D. S. Knitt, E. Doherty, M. Engelhardt, J. A. Doudna, and D. Herschlag, unpublished results). Thus, RNA may compensate for limited packing within a folded core via the addition of peripheral domains that pack onto and stabilize correct folding of the core. Alternatively, catalytic RNA cores may be stabilized by binding of a protein cofactor (*84*, *121*−*126*).

Why Structural Redundancy? Redundancy implies an absence of function, the antithesis of what is expected for a biological system governed by natural selection. What then could be the role or origin of the structural redundancy observed in protein enzymes and larger ribozymes? In other words, why have they evolved to form structures that are apparently impervious to most mutations? Some possible explanations are as follows. First, structural redundancy may provide a protection from denaturation and rapid degradation or the formation of undesirable interactions in vivo. It is also possible that structural redundancy arose over evolutionary time as a mechanism to ensure evolvability (*127*−*129*). In other words, it has been suggested that the ability to evolve new genes and functions provides a selective advantage in the long term. Structural redundancy can allow a protein or RNA to explore more of the sequence space, allowing for the possibility of encountering new or improved functions. In this context, the protelytic degradation of marginally stable proteins, which is important for housekeeping and control, could have also served in the course of evolution to ensure structural redundancy and thus evolvability. Evolvability could have provided part of the selective advantage for the evolution of degradative mechanisms or could have arisen as a secondary consequence of evolutionary pressure to develop these degradative mechanisms for removal of unfolded proteins. Another possibility is that small catalytic benefits of the wild-type sequences are large enough to allow a significant selective advantage over many generations, but that these catalytic benefits are too small to detect in standard enzymatic assays. Finally, it is possible that the portions of a protein or RNA not required for catalysis serve other roles, such as the formation and prevention of interactions with certain other macromolecules.

In summary, the hammerhead ribozyme is unable to efficiently position catalytic groups in the ground state. Rather, it must undergo an unfavorable folding transition to adopt its active conformation. Once folded, this small RNA enzyme may further lack the interconnections required to provide precise positioning and optimal catalysis. In contrast, protein enzymes of comparable size can achieve folding and efficient positioning through formation of extensive networks of interactions. Large RNA enzymes appear to compensate for their structural limitations by coopting peripheral folding domains and protein cofactors. These observations underscore the basic interrelationship, common to both RNA and protein systems, between folding and catalysis (*95*).

[9] The substantial gap between the catalytic proficiency of the hammerhead ribozyme and that of larger ribozymes, RNase P or the *Tetrahymena* group I ribozyme, is in agreement with the conclusions presented here. However, this comparison is limited because the larger ribozymes catalyze phosphoryl transfer to an intermolecular acceptor, whereas the hammerhead and other small ribozymes catalyze intramolecular attack by the 2′-hydroxyl to give a cyclic phosphate product.

ACKNOWLEDGMENT

We thank F. Wincott and A. DiRenzo for preparation of the oligonucleotides and A. Feig and O. Uhlenbeck for permission to cite unpublished results. We also thank G.

Narlikar for helpful discussions and the Herschlag lab for comments on the manuscript.

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BI980867Y