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

Alessio Peracchi, Alexander Karpeisky, Lara Maloney, Leonid Beigelman, and Daniel Herschlag

Department of Biochemistry, B400 Beckman Center, Stanford University, Stanford, California 94305-5307, and Ribozyme Pharmaceuticals Inc., 2950 Wilderness Place, Boulder, Colorado 80301
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 C 1 1.1, 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 (a), except for that for position 16.1, which was investigated in this study. kl is defined as k_{basic}/k_{wt}; k_{basic} and k_{wt} 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'-32P 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 of 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'-32P 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 MgCl2 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).

Control reactions in which the concentration of ribozyme was varied indicated that the substrate was completely bound in all cases so that k2, 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 k2 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 ≤ 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).
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., $K_i = K_d^{eq}$, Scheme 1) (25).

For $K_i$ to reflect $K_d^{eq}$ (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 (~14 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 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).

![Diagram of core folding model]

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 ($T_p$), 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.

![Graph of temperature dependence][1]

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). Further support for a distinction between the hammerhead and large RNA enzymes comes from the

![Graph of temperature dependence of cleavage reaction]

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, 101, 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.