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Structure–function relationships in the hammerhead ribozyme probed by base rescue

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ABSTRACT

We previously showed that the deleterious effects from introducing abasic nucleotides in the hammerhead ribozyme core can, in some instances, be relieved by exogenous addition of the ablated base and that the relative ability of different bases to rescue catalysis can be used to probe functional aspects of the ribozyme structure [Peracchi et al., *Proc Nat Acad Sci USA 93*:11522]. Here we examine rescue at four additional positions, 3, 9, 12 and 13, to probe transition state interactions and to demonstrate the strengths and weaknesses of base rescue as a tool for structure–function studies. The results confirm functional roles for groups previously probed by mutagenesis, provide evidence that specific interactions observed in the ground-state X-ray structure are maintained in the transition state, and suggest formation in the transition state of other interactions that are absent in the ground state. In addition, the results suggest transition state roles for some groups that did not emerge as important in previous mutagenesis studies, presumably because base rescue has the ability to reveal interactions that are obscured by local structural redundancy in traditional mutagenesis. The base rescue results are complemented by comparing the effects of the abasic and phenyl nucleotide substitutions. The results together suggest that stacking of the bases at positions 9, 13 and 14 observed in the ground state is important for orienting other groups in the transition state. These findings add to our understanding of structure–function relationships in the hammerhead ribozyme and help delineate positions that may undergo rearrangements in the active hammerhead structure relative to the ground-state structure. Finally, the particularly efficient rescue by 2-methyladenine at position 13 relative to adenine and other bases suggests that natural base modifications may, in some instance, provide additional stability by taking advantage of hydrophobic interactions in folded RNAs.

Keywords: abasic sites; binding; catalysis; chemical rescue; enzyme; functional groups; mutagenesis; ribozyme; RNA

INTRODUCTION

The hammerhead (Fig. 1) is the smallest known naturally occurring ribozyme and the only ribozyme for which a complete crystal structure has been determined (Pley et al., 1994; Scott et al., 1995, 1996; Murray et al., 1998), rendering it a prototype for the study of structure–function relationships in RNA. A large number of site-specific modifications have been made in the conserved core of this ribozyme, and the functional effects of these modifications have been probed (reviewed by Bratty et al., 1993; McKay, 1996; Birikh et al., 1997).

We showed previously that removal of the base at each of 14 positions in the hammerhead core, via substitution with a reduced abasic nucleotide (Fig. 2A), substantially decreases catalysis and that the deleterious effect can be alleviated at certain positions by exogenous addition of the removed base (Peracchi et al., 1996). Detailed analysis of "base rescue" for one hammerhead variant, bearing an abasic nucleotide at position 10.1 of the core, strongly suggested the exogenous rescuing base binds to the abasic site similarly to the originally removed guanine, utilizing Watson–Crick hydrogen bonding and stacking interactions, and, further, provided functional evidence for metal ion coordination (Peracchi et al., 1996; Peracchi et al., 1997; A. Peracchi, L. Beigelman & D. Herschlag, unpubl. results). This suggests that base rescue can be used as

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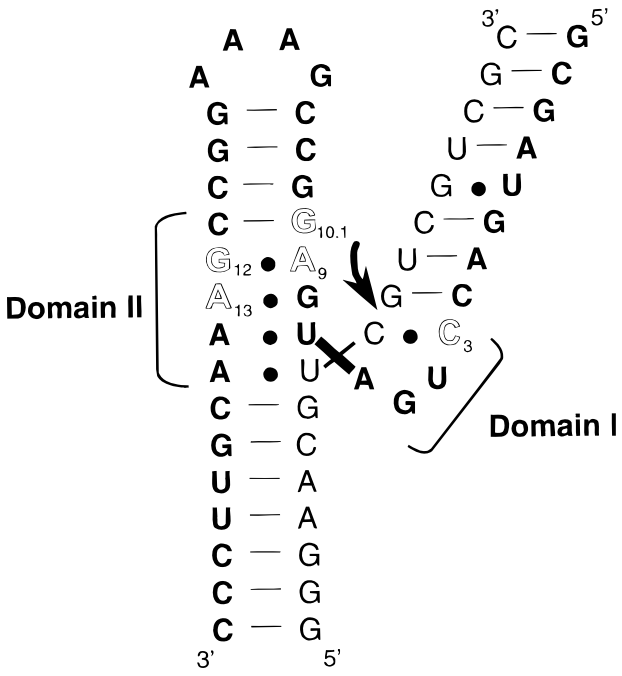


FIGURE 1. Structure of the hammerhead ribozyme HH16. The ribozyme is shown in bold and the substrate in thin letters. The secondary structure of the ribozyme is depicted schematically as observed in three dimensions (Pley et al., 1994; Scott et al., 1995, 1996; Murray et al., 1998). Nucleotides are numbered according to the standard hammerhead nomenclature (Hertel et al., 1992) and the five positions rescued by exogenous base addition with abasic constructs are outlined. The arrow indicates the position of cleavage. Domain I and domain II are defined as apparently distinct regions in the ground-state crystal structure (Pley et al., 1994; Scott et al., 1995, 1996; Murray et al., 1998).

a noncovalent site-specific modification approach, allowing multiple modifications at a single site to be rapidly tested without the need to synthesize a large number of oligonucleotides.

Here we describe structure–function analyses from base rescue experiments at four additional rescuable positions within the hammerhead core. These analyses are supplemented by the results from substitution with phenyl nucleotides (Fig. 2B) at the same positions. The results provide new insights into the functional interactions of the hammerhead ribozyme. They also help delineate the power and the limitations of base rescue in structure–function studies of ribozymes and other RNAs.

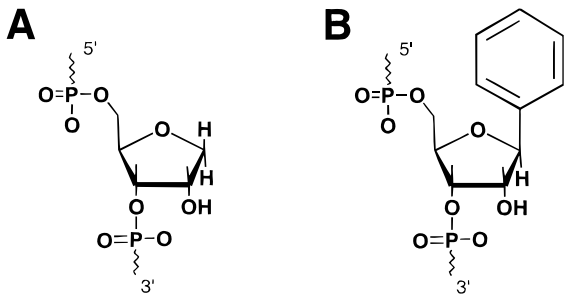


FIGURE 2. Structures of the modified nucleotides used in this study. **A**: A reduced abasic residue (1-deoxy-1-β-D-ribofuranose nucleotide). **B**: A phenyl nucleotide (1-phenyl-1-β-D-ribofuranose nucleotide).

RESULTS AND DISCUSSION

A simple model describing how exogenous bases can provide rescue at an abasic site is depicted in Figure 3 (Peracchi et al., 1996). According to this model, removal of a base interferes with the ability of the hammerhead core to achieve its catalytic structure. However, binding of the exogenous base can allow the active ribozyme structure to be achieved, thereby enhancing substrate cleavage ($k_2' > k_2$). Results supporting the model include the following (Peracchi et al., 1996): (1) The added bases have no significant effect on activity of the wild-type ribozyme; (2) there is specificity for the rescuing base, and this specificity is consistent with the structure and with previous structure–function analyses (see also below); and (3) a site-specific mutation of the ribozyme switched the specificity of base rescue, as predicted for Watson–Crick base pairing.

The increase in the observed rate constant for substrate cleavage by an abasic ribozyme variant (k_2^{obs}) with increasing exogenous base is described by Equation 1, which was derived from Figure 3 (Peracchi et al., 1996).

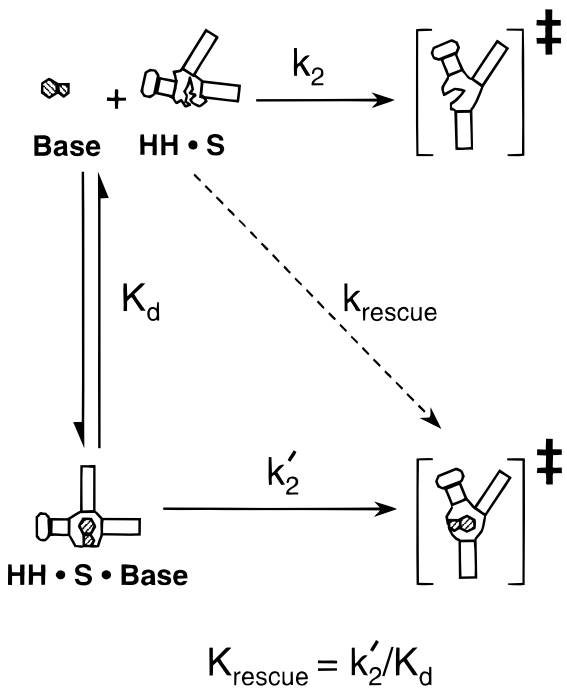


FIGURE 3. Minimal model for base rescue of abasic ribozymes (Peracchi et al., 1996).

$$k_2^{obs} = \frac{(k_2 + k_{rescue}[Base])}{1 + [Base]/K_d} \quad (1)$$

k_2 is the cleavage rate constant in the absence of base, K_d is the dissociation constant of the base, k_2' is the cleavage rate constant for the ternary ribozyme·substrate·base complex, and $k_{rescue}(=k_2'/K_d)$ is the apparent second-order rate constant for the rescue.

In the base rescue experiments described herein, the reaction rate increased with increasing base concentration, allowing k_{rescue} to be determined, but clear saturation was generally not observed even at base concentrations near their solubility limit, which prevented determination of k_2' and K_d. Measurement of k_{rescue} alone does not distinguish interactions that are present in the ribozyme·substrate·base ground state from those that are formed in the transition state; however, this renders k_{rescue} insensitive to nonproductive binding interactions, as these incorrect interactions are formed only in the ground state with bound base but are not present in the transition state (Fersht, 1985).

Another important strength of base rescue relative to traditional mutagenesis is that base rescue has the potential to reveal all of the transition state interactions formed by a given base. This is because k_{rescue} depends on all of the interactions that are made in going from ribozyme·substrate and free base to the ternary ribozyme·substrate·base complex in the transition state, as depicted by the dashed arrow in Figure 3. In contrast, functional groups with interactions that contribute to the overall structure are not revealed by traditional mutagenesis, unless their removal results in a rearrangement significant enough to perturb the active site. This point is further explained with base rescue results presented below (see Structural redundancy at position 9 and Fig. 7).

In the following sections, the rescue of four abasic variants of the HH16 hammerhead is analyzed. Rescue by differently modified pyrimidines and purines has been measured to ascertain the involvement of specific base functional groups in achieving catalysis. (For reference, the structures of a series of pyrimidine and purine bases are presented in Figure 4, with their respective numbering systems.) The analysis of these results is augmented by comparison of the effects of abasic and phenyl substitution at several positions; these effects are summarized in Table I and referred to throughout the text.

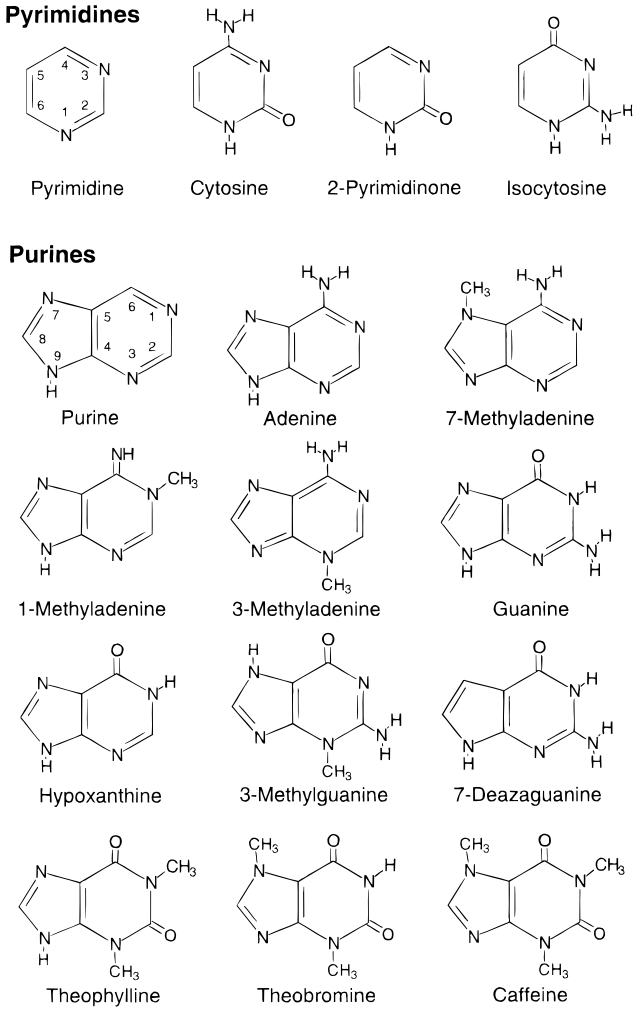


FIGURE 4. Structure and numbering system for some of the bases used in this study. For each base, the structure reported represents the tautomeric species most stable in aqueous solution as reported by Kwiatkowski & Pullman (1975) and Pullman & Pullman (1972). Protonation of 3-methylguanine at N7 was observed in the crystal structure of this base (Abola & Abraham, 1976). 7-Deazaguanine (2-amino-pyrrolo[2,3-d]pyrimidine-4(3*H*)-one) is included with the purines.

Specificity of rescue at position 3

C3 is a conserved residue that the hammerhead secondary structure fixes close to the cleavage site (Fig. 1) (Forster & Symons, 1987). Removal of the base at this position to give the C3*X* variant (*X* indicates an abasic residue) decreases catalysis 10^5-fold (Peracchi et al., 1996) (Table 1). Nevertheless, the functional importance of C3 is not obvious from the X-ray structures as this base is involved in only one hydrogen bond (Pley et al., 1994; Scott et al., 1995) (Fig. 5).

The deleterious effect of the abasic substitution at position 3 can be partially relieved by exogenous addition of cytosine. Addition of 50 mM cytosine, a concentration near the solubility limit of this base, provides a rescue of 20-fold for the C3*X* variant without affecting the wild-type reaction (Peracchi et al., 1996). To better understand the molecular basis of this activation, we investigated the rescue of the C3*X* variant by 14 different bases (Table 2). A strict specificity of rescue was observed, with purine bases and uracil giving no observable activation of the C3*X* mutant. In addition, removing or blocking the potential hydrogen bonding groups from the cytosine pairing face impaired rescue:

TABLE 1. Effect of abasic and phenyl substitutions in the HH16 core.

Nucleotide	k_{rel} for variants: Abasic	k_{rel} for variants: Phenyl	Ratio (Phenyl/Abasic)
C3	10^{-5}	2×10^{-5}	2
U4	0.07	0.02	0.3
A9	4×10^{-4}	0.055	130
G10.1	0.04	0.09	2.3
C11.1	0.16	0.06	0.25
G12	2×10^{-4}	10^{-4}	0.5
A13	$<5 \times 10^{-6}$	5×10^{-4}	>100
A14	$<5 \times 10^{-6}$	4×10^{-4}	>80
A15.1	$<5 \times 10^{-6}$	$<5 \times 10^{-6}$	—

k_{rel} is defined as $k_2^{variant}/k_2^{wt}$ and equals 1 for the wild-type ribozyme; k_2^{wt} and $k_2^{variant}$ are the first-order rate constants for the cleavage of the substrate in the complex with the wild-type and variant ribozyme, respectively (50 mM Tris-HCl, pH 7.5 and 10 mM $MgCl_2$ at 25 °C. See Methods for details). Under these conditions, $k_2^{wt} = 1.1 \pm 0.2$ min^{-1} (Peracchi et al., 1996) and the rate of the uncatalyzed reaction is $\sim 10^{-6}$ min^{-1} (Hertel et al., 1997). The effects of abasic modifications on the chemical step were reported previously (Peracchi et al., 1996) and are listed here for comparison.

4-aminopyrimidine (lacking the 2-keto group), 2-pyrimidinone (lacking the 4-amino group) and 3-methylcytosine each gave no observable activation. In contrast, efficient rescue was observed with isocytosine, which retains the same hydrogen bonding groups as cytosine (Fig. 4). These results suggest that the modest rescue at position 3 requires either cytosine or a similar pyrimidine base bearing the same hydrogen bonding groups on the pairing face (Equation 2, groups in bold).

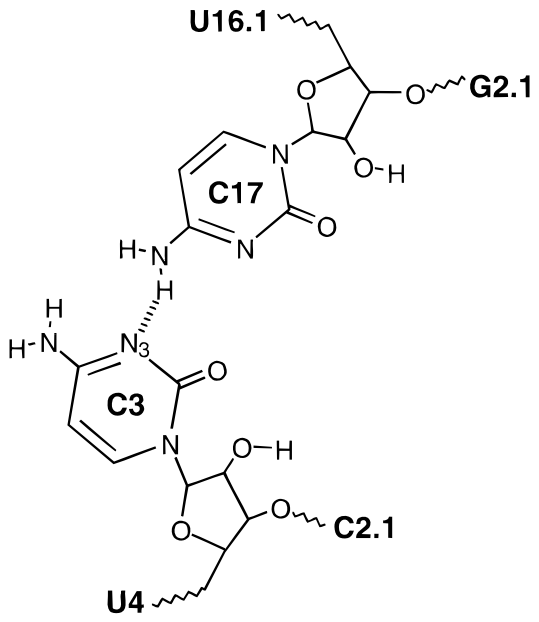


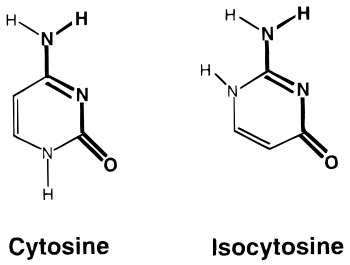
FIGURE 5. Schematic representation of the hydrogen bonding interactions of the C3 base observed in the ground-state crystal structures of the hammerhead ribozyme (Pley et al., 1994; Scott et al., 1995).

TABLE 2. Base rescue of the C3*X* variant.

Base	k_{rescue}[a] (10^{-3} M^{-1} min^{-1})	k_{rescue}^{rel}[b]
Cytosine	3.5 ± 0.5	(1)
4-Aminopyrimidine	≤0.2	≤0.05
2-Pyrimidinone	≤0.2	≤0.05
3-Methylcytosine	≤0.2	≤0.05
1-Methylcytosine	12 ± 2	3.5
2-Thiocytosine	5 ± 2.5	1.4
Isocytosine	3.5 ± 1.5	1
6-Methylisocytosine	4 ± 0.5	1.1
Uracil	≤0.2	≤0.05
Cytidine	≤0.2	≤0.05
Adenine	≤1	≤0.2
Guanine	≤50	≤15
7-Deazaguanine	≤5	≤1.4
2,6-Diaminopurine	≤1	≤0.2

[a] The values of k_{rescue} were obtained as described in Materials and Methods. When errors are indicated, the k_{rescue} values represent the average of at least two independent determinations. Upper limits of k_{rescue} are given for bases that did not provide significant activation (<1.5-fold at the highest base concentration used).

[b] k_{rescue}^{rel} for a given base is defined as $k_{rescue}/k_{rescue}^{Cytosine}$. Rescue by cytosine is used as the reference and denoted in parentheses because it is the base originally removed.



(2)

A role for these functional groups is consistent with the observation that replacing the C3 base with a simple phenyl ring (Fig. 2B), bearing no hydrogen bonding groups, is essentially as deleterious as replacing C3 with an abasic nucleotide (Table 1). Nevertheless, it should be recognized that the maximal rescue observed for the C3*X* variant gives a rate that is nearly 10^4-fold slower than that of the wild-type ribozyme and that caution is required in interpreting base rescue results in cases of modest rescue.

C3: New interactions in the transition state?

Of the three groups on the pairing face of C3, only N3 appears to be engaged in a hydrogen bond in the crystal structure of the ribozyme (Fig. 5). The base rescue results suggest that the exogenous base forms additional interactions in the transition state, and imply that this may also occur for C3 in the wild-type ribozyme. The results in Table 2 are in agreement with the previous observation of an eightfold decrease in rate of

substrate cleavage upon converting the cytosine at position 3 to 2-pyrimidinone (Murray et al., 1995), which suggested a functional role for the 4-amino group. The base rescue results are further consistent with C3 forming additional transition state interactions that also involve the 2-keto group and N3 of the pyrimidine ring (Equation 2, groups in bold). It is also possible that N3 is involved in a different interaction in the transition state than in the ground state, as the hydrogen bond observed in the initial X-ray structures (Pley et al., 1994; Scott et al., 1995) (Fig. 5) is absent in the structure of a ribozyme-RNA substrate complex obtained at high pH (Scott et al., 1996).

There is growing evidence for a conformational transition preceding cleavage of bound substrate by the hammerhead ribozyme. It was noted that the X-ray structures of hammerhead complexes would have to rearrange to allow an in-line nucleophilic attack at the cleavage site (Pley et al., 1994; McKay, 1996). Further, several groups lacking substantial interactions in these ground-state structures and in structures with rearrangements around the cleavage site (Pley et al., 1994; Scott et al., 1995, 1996; Murray et al., 1998) are critical for catalysis (McKay, 1996; Baidya et al., 1997), suggesting that the rearrangement may be extensive. In addition, a metal ion located ~20 Å away from the cleavage site was shown to be crucial for catalysis and likely to adopt at least one additional ligand in the transition state, again consistent with a large scale conformational change prior to catalysis (Peracchi et al., 1997). The transition state interactions with specific groups on the C3 base ring implied from the base-rescue experiments, contrasted with the X-ray structures (Pley et al., 1994; Scott et al., 1995, 1996; Murray et al., 1998) (Fig. 5), provide additional evidence for a conformational rearrangement and may ultimately contribute to a molecular understanding of the functional conformation.

Efficient rescue by adenine at position 9

The three-dimensional structure of the hammerhead ribozyme shows that the adenine base at position 9 is involved in a nonstandard base pair with G12, and that this pair is stacked between a Watson–Crick base pair (G10.1·C11.1) and another nonstandard pair (G8·A13) (Fig. 6; Pley et al., 1994; Scott et al., 1995). The A9·G12 pair involves three hydrogen bonds, two with the exocyclic amino group of the adenine and one with N7 of this base (Fig. 6). We previously showed that removing the A9 base in the HH16 hammerhead core decreases catalysis by 2,000-fold (Peracchi et al., 1996) (Table 1) and that exogenous addition of 3 mM adenine activates this abasic variant by 300-fold, to within 10-fold of the wild-type rate (Peracchi et al., 1996).

Table 3 summarizes the results from rescue experiments of A9*X* with 28 different bases. Rescue was specific for purines compared to pyrimidines. Furthermore,

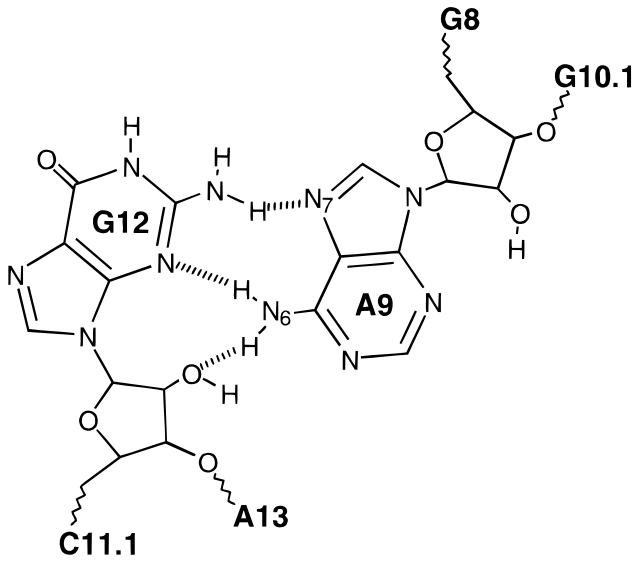


FIGURE 6. Schematic representation of the hydrogen bonding interactions of the A9 base observed in the crystal structures of the hammerhead ribozyme (Pley et al., 1994; Scott et al., 1995, 1996; Murray et al., 1998).

K_{rescue} for adenine was ~30-fold higher than for purine itself, suggesting a functional interaction with the 6-amino group of the rescuing base. In addition, methylation of the adenine ring at position 7 decreases k_{rescue} by ~fivefold. This is consistent with disruption of an interaction of N7 in the transition state, although a steric effect cannot be excluded. Rescue by 3-methyladenine is similar to that by unmodified adenine, and rescue by 2-methyladenine is even better, suggesting that there are no functional interactions at positions 2 and 3 in the transition state of the wild-type ribozyme. Previous site-specific mutagenesis studies, in which A9 had been modified to 1-deazaadenine and 3-deazaadenine, suggested that N1 and N3 do not engage in a critical transition state interaction (Bevers et al., 1996; Seela et al., 1998).

Overall, although the effects on base rescue from modifying the adenine functional groups are modest, the data above are consistent with A9 forming the same interactions in the transition state that are observed in the ground-state crystal structure, without the formation of new functional interactions. Given the accumulating evidence for a substantial precatalytic rearrangement of the hammerhead core structure discussed above, it is important to map positions that do and do not take on additional transition state interactions.

Replacement of the A9 base by an unsubstituted phenyl ring (to give variant A9$\underline{\Phi}$, with $\underline{\Phi}$ indicating the phenyl nucleotide derivative shown in Fig. 2B) decreases catalysis by a modest 20-fold compared to the 2,000-fold deleterious effect from removal of the base (Table 1). Such effectiveness of the phenyl substitution

TABLE 3. Base rescue of the A9*X* variant.

Base	k_{rescue}[a] (M^{-1} min^{-1})	k^{rel}_{rescue}[b]
Purines bearing no amino groups		
Purine	3.2 ± 0.6	0.03
Hypoxanthine	6.5 ± 3	0.06
Xanthine	<1.5	<0.02
Theobromine	10 ± 3	0.1
Theophylline	0.6 ± 0.3	0.006
Caffeine	16 ± 3	0.15
Purines with a 6-amino group		
Adenine	105 ± 45	(1)
1-Methyladenine	23 ± 2	0.22
2-Methyladenine	460 ± 70	4.5
3-Methyladenine	85 ± 30	0.8
7-Methyladenine	16 ± 2	0.17
2,6-Diaminopurine	160 ± 30	1.5
Adenosine	2	0.02
Purines with a 2-amino group		
2-Aminopurine	90	0.9
Guanine	610 ± 150	5.8
6-Thioguanine	600 ± 250	5.8
1-Methylguanine	160 ± 60	1.5
3-Methylguanine	13 ± 2	0.12
7-Deazaguanine	750 ± 200	7.1
7-Methylguanine	2	0.02
8-Hydroxyguanine	25	0.25
8-Mercaptoguanine	25	0.25
Guanosine	18 ± 7	0.17
Guanosine 2′3′ acyclic dialcohol	2.7 ± 0.5	0.03
Pyrimidines		
Uracil	0.04	4×10^{-4}
Cytosine	0.04	4×10^{-4}
6-Methyl Isocytosine	0.11	1×10^{-3}
Other ring systems		
Pterin	≤20	≤0.2
Indole	0.9 ± 0.1	0.01

[a]The values of k_{rescue} were obtained as described in Materials and Methods. When errors are indicated, the k_{rescue} values represent the average of at least two independent determinations. Upper limits of k_{rescue} are given for bases that did not provide significant activation (<1.5-fold at the highest base concentration used).

[b]k^{rel}_{rescue} for a given base is defined as $k_{rescue}/k^{Adenine}_{rescue}$. Rescue by adenine is used as the reference and denoted in parentheses because it is the base originally removed.

relative to abasic substitution is observed at only three of the nine positions in the HH16 core at which both modifications were tested (Table 1).

What is the role of the A9 base in hammerhead catalysis? The large effect of base ablation (Peracchi et al., 1996) coupled with the apparent absence of new interactions in the transition state suggests that the primary role of the A9 base is to ensure the positioning of its neighbors. The efficient reaction of A9$\underline{\Phi}$ suggests that stacking of A9 may provide a spacer to maintain positioning within domain II of the hammerhead core and prevent structural collapse. This base may be required to preserve the integrity and positioning of a nearby metal–ion-binding site that appears to be critical for catalysis (Peracchi et al., 1997).

Structural redundancy at position A9

Even though the 6-amino group and the N7 atom from A9 are involved in interactions in the X-ray structures (Fig. 6; Pley et al., 1994; Scott et al., 1995, 1996; Murray et al., 1998), removal of either group from an intact hammerhead did not impair catalysis (Fu & McLaughlin, 1992a,b; Slim & Gait, 1992). In contrast, the rescue experiments described above suggest that these interactions have functional significance in the chemical step.

To understand this paradox, consider a nucleotide base that is positioned by multiple ground-state interactions that are maintained in the transition state (Fig. 7A). If, upon removal of one of the interacting functional groups by chemical mutagenesis, the other interactions are sufficient to maintain positioning for the transition state, then no effect on the reaction rate would be observed (Fig. 7A, $k = k'$). Thus, even though the functional group being probed is involved in an interaction in the transition state, traditional functional group mutagenesis would be blind to this interaction, giving the same readout as functional groups that make no interactions in the transition state. In contrast, in a base rescue experiment, removal of the same interaction would have an effect because one less interaction is formed in the transition state (Fig. 7B, $k_{rescue} > k'_{rescue}$). Thus, functional interactions not identified by traditional mutagenesis because of local structural redundancy can be revealed by effects on k_{rescue} in the base-rescue approach. The 6-amino group and N7 of A9 may provide two examples.

Promiscuity of rescue and alternative binding modes at position 9

A9 is a conserved residue in natural hammerheads (Forster & Symons, 1987), and mutating it to G produces a 100-fold decrease in catalysis (Ruffner et al., 1990; Ng et al., 1994). Furthermore, since guanine lacks the 6-amino group of adenine, it is unable to make the transition state interactions implicated for adenine (Fig. 6). It was therefore surprising to observe that guanine gave better rescue of the A9*X* variant than adenine (Table 3 and Fig. 8A).

The data can be reconciled by invoking a model in which exogenous guanine can rescue A9*X* by adopting the alternative binding mode depicted in Figure 8B. In this binding mode, the 2-amino group forms two hydrogen bonds with the ribozyme substituting for the 6-amino group of adenine (Fig. 6), while N3, instead of N7, is involved in the third hydrogen bond. Consistent with this model, the 100-fold-better rescue with guanine than with hypoxanthine indicates that the 2-amino group contributes substantially to rescue (Table 3). A role for N3 is implied by the 50-fold lower k_{rescue} of 3-methylguanine compared to guanine (Table 3). Also consistent with

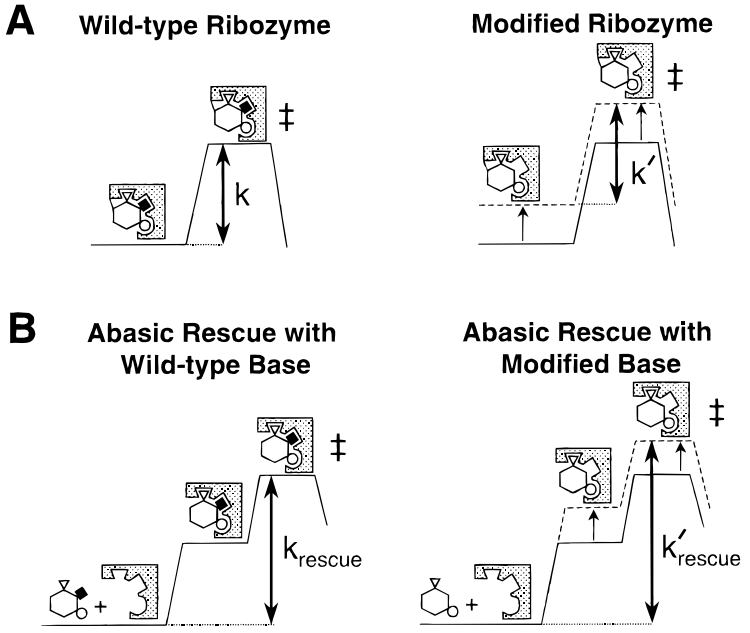


FIGURE 7. The effects of probing a redundant functional interaction via site-directed mutagenesis (**A**) or base rescue (**B**), described by free energy reaction profiles. **A**: For the wild-type ribozyme, the interaction in question is present in both the ground state and in the transition state. Removal of the interacting functional group of the base (■) destabilizes the ground state and the transition state to the same extent because, in this model, the remaining redundant interactions are sufficient to maintain the local structure. Thus, the experiment does not reveal whether the functional group removed is involved in an interaction in the transition state ($k = k'$). **B**: A base rescue experiment probing the same interaction. Removal of the interacting functional group of the base is deleterious to rescue ($k_{rescue} > k'_{rescue}$). This is because the base is free in the ground state but bound in the transition state, so that each interaction between the base and the ribozyme contributes to k_{rescue}, as further described in the text.

the model in Figure 8B are the observations that N1 of guanine can be methylated without a large decrease in k_{rescue} and that the nature of the 6-substituent is relatively unimportant in bases containing a 2-amino group. For example, 2,6-diaminopurine and 2-aminopurine have rescuing abilities within twofold of one another (Table 3).

The similar k_{rescue} values by guanine and 7-deazaguanine provide no indication of a role for N7 in rescue by guanine. Nevertheless, methylation at the 7 position has a large deleterious effect on rescue of ~300-fold. This could arise from introduction of steric clash. In general, addition of groups on the imidazole moiety of guanine affects rescue significantly: 8-hydroxyguanine and 8-mercaptoguanine, guanosine and guanosine 2′,3′-acyclic dialcohol (modified at position 9), and pterin, in which the imidazole ring of guanine is replaced by a larger pyrazine ring, all showed markedly decreased k_{rescue} values. These effects are most simply attributable to steric hindrance with the ribozyme core.

The alternative binding modes that allow rescue by adenine and guanine provide an example of promiscuous interactions in RNA. It has been suggested that promiscuity in RNA/RNA interactions is common, because of the limited repertoire of residue types and the preponderance of hydrogen bond donors and acceptors (Sigler, 1975; Herschlag, 1995). A predominant role of the residue at position 9 as a spacer, as suggested above, might render this position more prone to promiscuous binding relative to residues involved in more direct interactions with the groups undergoing chemical rearrangement.

Rescue at position 13

In the ground-state structure of the hammerhead, the A13 nucleotide forms a nonstandard G·A pair with G8 (Fig. 1). Pairing occurs via three hydrogen bonds, involving the exocyclic amino group and the N7 atom of A13 (Pley et al., 1994; Scott et al., 1995) (Fig. 9). Removal of the base from A13 essentially abolishes catalysis, reducing the cleavage rate by almost 10^6-fold (Table 1) (Peracchi et al., 1996). Addition of adenine to near its solubility limit (~3 mM) gives a modest rescue of ~30-fold (Peracchi et al., 1996).

A survey of rescue for A13*X* is reported in Table 4. Strikingly, many of the purines tested rival the rescuing efficiency of adenine, the base originally removed. Compounds containing an exocyclic amino group, such as adenine or 2-aminopurine, or a keto group, such as hypoxanthine, and purine, which lacks exocyclic groups, all show similar values of k_{rescue}. These observations with base rescue mirror the small effects observed previously with hammerhead constructs containing purine, 1-deazaadenosine, 3-deazaadenosine, 7-deazaadenosine, or isoguanosine substituted at position 13 (Fu & McLaughlin, 1992a; Slim & Gait, 1992; Fu et al., 1993; Seela et al., 1993; Bevers et al., 1996; Seela et al., 1998). Finally, the nucleosides adenosine and guanosine are surprisingly efficient in rescue at position 13 relative to the poor rescue observed with nucleosides at other positions (Tables 2–4; Peracchi et al., 1996), suggesting that a greater freedom of motion at position 13 minimizes steric clashes of the nucleoside and backbone sugars.

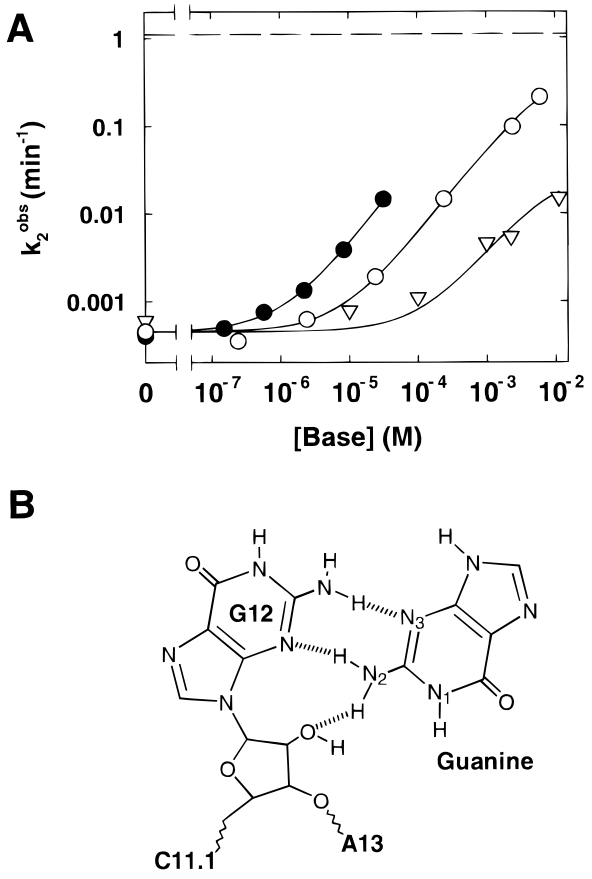


FIGURE 8. Efficient rescue of A9*X* by guanine. **A**: Activation of A9*X* cleavage by increasing concentrations of guanine (●), adenine (○) and purine (▽). Values of k_2^{obs} were measured under single-turnover conditions as described in Materials and Methods. The solid lines represent nonlinear least-squares fits to Equation 1. Some curvature is observed at the highest base concentrations, but this could arise from base aggregation or small additional inhibitory effects and is therefore not interpreted in the text. The dashed line indicates the wild-type rate (1.1 min^{-1}) and is shown for comparison. **B**: Proposed binding mode for rescue by guanine at position 9.

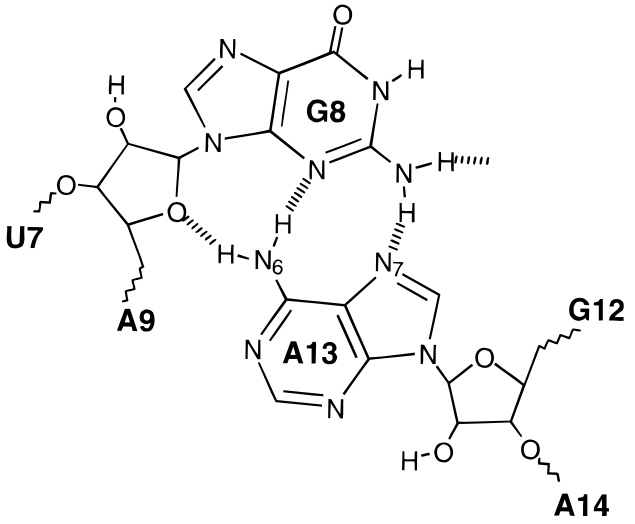


FIGURE 9. Schematic representation of the hydrogen bonding interactions of the A13 base observed in the ground-state crystal structures of the hammerhead ribozyme (Pley et al., 1994; Scott et al., 1995).

Whereas the constellation of hydrogen-bond donors and acceptors on the purine ring do not affect rescue significantly, the pyrimidines tested are at least 100-fold less effective in rescue than the purines (Table 4). Further, the HH16 variant A13$\underline{\Phi}$, in which the A13 base is replaced with an unsubstituted phenyl ring, is >100-fold more active than the A13*X* ribozyme, in which the base has been removed (Table 1). These observations suggest that stacking is crucial for rescue and are consistent with a role of the adenine base at position 13 in orienting nearby residues, analogous to the role proposed for A9.

Rescue at position 13 is only partial, however, as the observed cleavage rate for the A13*X* variant with the highest concentration of adenine used (3 mM), remains 10^4-fold slower than cleavage by the unmodified ribozyme. This is in contrast to the maximal observed rescue of the A9*X* variant by adenine, which gave a cleavage rate within 10-fold of wild type (Fig. 8A). The weaker rescue of the A13*X* variant could arise from weaker binding of the base, consistent with the recent proposal that the hydrogen bonds observed in the ground-state G8·A13 pair are broken in the transition state (Murray et al., 1998). Alternatively, formation of stable collapsed conformers of an abasic ribozyme can also impede rescue. Less efficient rescue could also arise if tethering of the base and sugar via the glyco-

TABLE 4. Base rescue of the A13*X* variant.

Base	k_{rescue}[a] ($M^{-1}min^{-1}$)	k_{rescue}^{rel}[b]
Adenine	0.11 ± 0.04	(1)
Purine	0.05 ± 0.01	0.5
Hypoxanthine	0.06 ± 0.02	0.6
1-Methyladenine	0.17 ± 0.08	1.6
2-Methyladenine	2.4 ± 0.6	22
3-Methyladenine	0.03 ± 0.003	0.3
2,6-Diaminopurine	0.03	0.3
2-Aminopurine	0.09 ± 0.03	0.9
Adenosine	0.02 ± 0.01	0.2
Guanine	≤0.05	≤1
7-Deazaguanine	0.11 ± 0.01	1
Guanosine	0.035 ± 0.005	0.3
Cytosine	$\leq 5 \times 10^{-4}$	≤0.005
Uracil	$\leq 5 \times 10^{-4}$	≤0.005

[a] The values of k_{rescue} were obtained as described in Materials and Methods. When errors are indicated, the k_{resuce} values represent the average of at least two independent determinations. Upper limits of k_{rescue} are given for bases that did not provide significant activation (<1.5-fold at the highest base concentration used).

[b] k_{rescue}^{rel} for a given base is defined as $k_{rescue}/k_{rescue}^{Adenine}$. Rescue by adenine is used as the reference and denoted in parentheses because it is the base originally removed.

sidic bond is necessary to ensure precise positioning within the transition-state structure. Similarly, a functional group modification can sometimes have a smaller deleterious effect in base rescue than in site-specific modification, because the alignment of the cognate base may be suboptimal in base rescue and the absence of a covalent tether will allow the rescuing base more freedom to rearrange to avoid unfavorable interactions. An example of this may be the 20-fold deleterious effect from the site-specific modification of A13 to 1-deazaadenosine contrasted with the similar rescue by 1-methyladenine and adenine (Seela et al., 1998; Table 4).

Given the similar rescue at position 13 by a variety of purines, it was particularly surprising that 2-methyladenine exhibited 22-fold greater rescue than adenine. This enhanced rescue, corresponding to a contribution of 1.8 kcal/mol toward transition state stabilization, presumably arises from formation of a fortuitous hydrophobic interaction within the core that could involve stacking and/or additional interactions. It has been suggested that base and sugar modifications in tRNA and rRNA may function in structural stabilization (Hall et al., 1989; Perret et al., 1990; Kintanar et al., 1994; Yue et al., 1994; Ushida et al., 1996; G.D. Glick & E.J. Maglott, unpubl. results). The substantial enhancement of rescue by 2-methyladenine suggests that some of these modifications could stabilize folded RNAs by hydrophobic interactions. Methylation at the 2 position of adenine is one of several naturally occurring RNA modifications that increase hydrophobicity (Agris, 1996).

Rescue at position 12 by a soluble guanine analog

In the hammerhead crystal structure, the base of G12 is positioned by four hydrogen bonds to G8 and A9 in the opposite strand and stacks onto the G10.1·C11.1 base pair (Pley et al., 1994; Scott et al., 1995, 1996; Murray et al., 1998). Despite this, and in contrast with the rescue observed at the adjacent positions 9, 10.1, and 13, the hammerhead variant G12*X* is not rescued by the addition of exogenous guanine (Peracchi et al., 1996). However, the solubility of guanine is low (~30 μM, which is 200-fold less than adenine and 2,000-fold less than cytosine). A more soluble guanine analog, 7-deazaguanine, gave a small rate increase with the G12*X* variant (threefold at 2 mM base), without increasing the wild type reaction (data not shown). The ability to observe some rescue with 7-deazaguanine is consistent with the efficient catalysis observed with a hammerhead construct containing 7-deazaguanosine at position 12 (Fu et al., 1993), although the small extent of rescue precluded a detailed characterization. Nevertheless, the use of soluble base analogs may sometimes allow the use of base rescue to probe additional abasic sites.

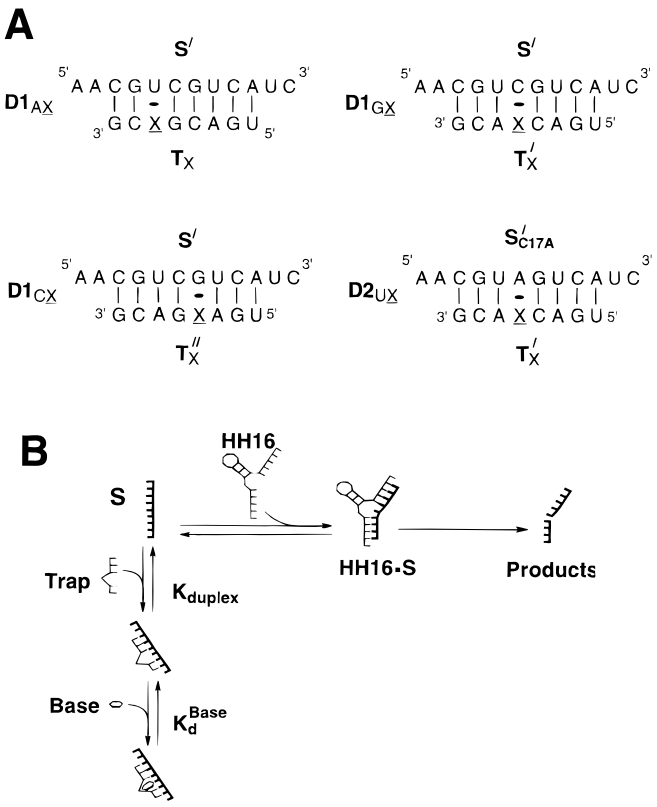
Stabilization of a model RNA duplex by exogenous base addition

In catalytic RNAs like the hammerhead ribozyme, observation of base rescue does not require saturation by the exogenous base; even a small fraction of the total ribozyme in the active RNA-base complex can be enough to substantially enhance the observed reaction rate (Fig. 2). In contrast, for RNAs with functions that are assayed as equilibrium events, rescue may only be observable if a substantial fraction of the RNA is complexed with base; that is, a twofold signal would require half of the RNA to be complexed with the exogenous base.

To determine how difficult it is to saturate an abasic site, the stability of a series of abasic-containing duplexes (Fig. 10A) was determined in the absence and in the presence of a base complementary to the residue across from the abasic site. If the exogenous base could give saturation, it would stabilize the RNA duplex, that is, increase the observed affinity of the two RNA strands. Because one of the two strands used in this experiment was an HH16 substrate, stabilization of the duplex would inhibit the cleavage reaction, thereby providing a readout for base binding (Fig. 10B).

However, adenine, guanine, cytosine and uracil at concentrations near their solubility limit (2.6, 0.02, 46 and 30 mM, respectively) did not stabilize duplex $D1_{AX}$, $D1_{GX}$, $D1_{CX}$ and $D2_{UX}$, respectively. That is, the inhibition by the abasic-containing strand was not significantly increased upon base addition (±twofold effect, data not shown; see Methods for experimental details). Thus, the K_d^{Base} values for binding of these bases to the abasic duplexes are greater than the base concentrations used. Similarly, there was no clear evidence for saturation when the four natural bases were used to rescue the abasic hammerhead variants (Peracchi et al., 1996; see also above).

We therefore tested the ability of 2,6-diaminopurine to stabilize a duplex containing an abasic site facing a U. 2,6-Diaminopurine can stack well, is soluble up to ~10 mM, and can form a base pair with U containing three hydrogen bonds. 2,6-Diaminopurine produced a substantial decrease in the observed rate of cleavage of the substrate strand (Fig. 10C), consistent with base inhibition arising from stabilization of the duplex between the substrate and its complementary strand. The data in Figure 10C give a dissociation constant $K_d^{Base} = 0.3 \pm 0.1$ mM for binding of 2,6-diaminopurine to the abasic site facing a U in our model duplex. A similar value of K_d^{Base} 0.6 ± 0.2 mM was obtained for binding of 2,6-diaminopurine to the same duplex at 35 °C (data not shown). These values are close to the observed dissociation constant of 1.5 mM for 2,6-diaminopurine binding to G10.1*X*, the hammerhead construct with an abasic site across from a U residue at position 11.1 (Peracchi et al., 1996).



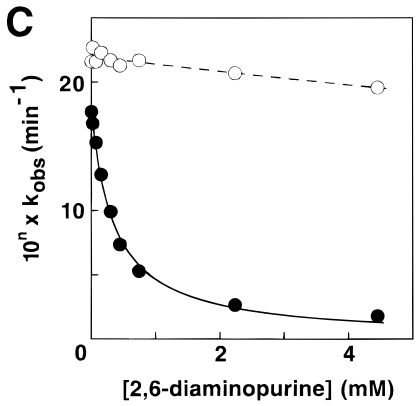


FIGURE 10. Use of substrate inhibition to determine stabilization of abasic RNA duplexes by free bases. **A**: The duplexes used in these experiments. Each duplex is formed between a short HH16 substrate (S′ or S'_{C17A}) and a complementary trap strand containing an abasic residue (T_X, T'_X or T''_X). **B**: Schematic representation of the substrate inhibition approach. The trap strand is added to allow duplex formation with the substrate (S). Binding of an exogenous base to the abasic site would drive the equilibrium toward formation of the duplex (vertical arrows), thereby reducing the concentration of free substrate and inhibiting the cleavage reaction (horizontal arrows; see Methods for details). **C**: Substrate inhibition of S′ cleavage as a function of [2,6-diaminopurine] in the presence of trap strand T_X (● $n = 3$) or in its absence (○ $n = 2$). Cleavage of S′ was followed at subsaturating ribozyme concentration (8 nM), in the presence of 2 μM T_X in 50 mM Tris-HCl, pH 7.5 and 10 mM $MgCl_2$ at 25 °C. The solid line through the data points represents the best fit to Equation 5 in Methods, yielding a $K_d^{Base} = 0.3 \pm 0.1$ mM for binding of 2,6-diaminopurine to duplex $D1_{AX}$. Normalization to account for the small amount of inhibition by the added base in the absence of trap strand had no significant effect (not shown).

The observation of base saturation within a model RNA duplex implies that the base rescue approach may occasionally be useful in the study of noncatalytic nucleic acids. Furthermore, it may be possible in some cases to amplify a signal from low-occupancy base binding by coupling binding to chemical or enzymatic modification of the RNA. Nevertheless, application of base rescue is particularly favored in the study of catalytic systems, because rescue can be observed in the absence of saturation of the binding site.

CONCLUSIONS AND IMPLICATIONS

Hammerhead structure–function

Using base rescue, we have probed transition state interactions of several functional groups in the hammerhead ribozyme core. The results allow models for interactions at several positions.

Analysis of rescue at position 3 has suggested that each of the groups on the base-pairing face of C3 is important for catalysis. Contrasting these results and the large effect from abasic and phenyl substitution at position 3 with the crystal structure suggests that this base forms interactions in the transition state that are not present in the ground state.

Conversely, the results of base rescue at position 9 are consistent with A9 retaining in the transition state the same interactions observed in the ground-state crystal structure. These results and the enhanced reactivity of a ribozyme with a phenyl nucleotide instead of an abasic at position 9 suggest that the main role of this base in the transition state is to engage in interactions that maintain positioning within domain II of the hammerhead core (Fig. 1).

The low efficiency and low specificity of rescue at position 13 prevent definitive conclusions about the role of groups on the A13 nucleotide. Nevertheless, the ability of many purines but not pyrimidines to partially rescue A13*X* and the enhanced reactivity of the ribozyme with a phenyl group instead of an abasic at position 13 suggest that A13, like A9, may act mainly via stacking interactions.

A13 stacks onto both A9 and A14 in the ground-state crystal structures of the hammerhead. The importance of stacking at position 14 can be inferred from the observation that variant A14$\underline{\Phi}$, bearing a phenyl nucleotide at position 14, is >80-fold more active than the

A14*X* ribozyme, in which the base has been removed (Table 1). Thus, it is reasonable that the stacking interactions of A9, A13 and A14 observed in the ground-state hammerhead structures are maintained in the transition state.

In summary, the results described herein lead to a working model in which these stacking interactions are used to orient other groups that are more directly involved in transition state stabilization.

Finally, the enhanced base rescue observed in one case at position 13 with a nonstandard methylated base suggests that small hydrophobic regions may exist in naturally occurring RNA structures. Some of the naturally occurring RNA modifications may take advantage of such interactions to provide more stable and more rigid RNA structures (e.g., Hall et al., 1989; Perret et al., 1990; Kintanar et al., 1994; Yue et al., 1994; Ushida et al., 1996; G.D. Glick & E.J. Maglott, unpubl. results).

Base rescue

The study of structure–function relationships in RNA entails determining the groups on a particular nucleotide that are important for function and, ultimately, why they are important. The traditional way to address this question is via site-specific mutagenesis or modification, but a thorough exploration of a single position by this technique requires the synthesis of many RNA variants, typically more than one variant for each group to be tested. Base rescue provides a potential alternative to traditional mutagenesis, akin to the chemical complementation approach adopted in protein studies to investigate the properties of active site and structural residues (Toney & Kirsch, 1989, 1992; Tu et al., 1989; Kim et al., 1990; Carter et al., 1991; Smith & Hartman, 1991; Zhukovsky et al., 1991; Eriksson et al., 1992; Phillips et al., 1992; Lu et al., 1993; Sekimoto et al., 1993; Barrick, 1994; Dhalla et al., 1994; Fitzgerald et al., 1994; Harpel & Hartman, 1994; Perona et al., 1994; Carlow et al., 1995; Hong et al., 1995; Morton & Matthews, 1995; Morton et al., 1995; Frillingos & Kaback, 1996; Newmyer & Ortiz de Montellano, 1996; Rynkiewicz & Seaton, 1996; Barrick et al., 1997; Boehlein et al., 1997; Huang & Tu, 1997; Kim et al., 1997). In the RNA field, Cernà and coworkers studied stimulation of the ribosomal peptidyl transfer reaction from a minimal substrate, 3′-*O*-(*N*-formylmethionyl) adenosine 5′-phosphate (pA-fMet), by exogenous cytosine and cytosine derivatives. Stimulation was attributed to the bases mimicking the interactions formed at the ribosome donor sites by CpCpA-fMet, which is a better substrate (Cernà, 1975; Cernà et al., 1978).

The base rescue approach requires the preparation of only one RNA derivative per position to be tested. An abasic residue can be introduced at the desired location via solid-phase synthesis in the case of small RNAs (Beigelman et al., 1994, 1995; Schmidt et al., 1996; Chartrand et al., 1997) or by semisynthetic techniques for large RNAs (e.g., see Abramovitz et al., 1996). A battery of commercially available bases and base derivatives can then be tested for rescue. Mutations cannot always be rescued; only 5 out of 14 abasic hammerhead variants were activated by exogenous bases. Nevertheless, "non-covalent mutagenesis" at a subset of positions can provide a fast and economical structure–function approach relative to standard mutagenesis. Furthermore, as outlined in Figure 7, base rescue in ribozymes has the potential to reveal all of transition state interactions formed by a given base, including interactions that can be obscured in traditional mutagenesis studies by structural redundancy. Among the limits of the base-rescue approach are the difficulty of achieving saturation of the abasic sites and the possibility of observing new fortuitous interactions for the exogenous bases, resulting in the adoption of binding modes that differ from those of the base originally removed.

MATERIALS AND METHODS

Materials

Oligonucleotides

The wild-type hammerhead ribozyme HH16 and its variants and substrates were prepared by solid-phase synthesis (Wincott et al., 1995). The introduction of reduced abasic nucleotides and 1-phenyl-β-D-ribose nucleotides in RNA produced by solid phase synthesis has been described (Beigelman et al., 1994, 1995; Matulic-Adamic et al., 1996). The hammerhead substrates were 5′-end labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase and purified by nondenaturing PAGE. Oligonucleotide concentrations were determined using specific activities for radioactive RNAs and assuming a residue extinction coefficient of 8.5×10^3 M^{-1} cm^{-1} for nonradioactive RNAs.

Chemicals

The bases and base derivatives used in the rescue experiments were of the best quality commercially available. 4-Aminopyrimidine, 2-pyrimidinone (2-hydroxypyrimidine), 6-methylisocytosine (2-amino-4-hydroxy-6-methylpyrimidine), 2,6-diaminopurine, 8-hydroxyguanine (2-amino-6,8-dihydroxypurine) and 8-mercaptoguanine (2-amino-6-hydroxy-8-mercaptopurine) were purchased from Aldrich; pterin and 6-thioguanine were from Lancaster; 3-methylguanine was from Fluka; 7-methyladenine was from Chemsyn Science Laboratories; all of the other bases were from Sigma. These compounds were not further purified; thus, low levels of rescue may represent upper limits for the rescuing ability of specific bases in certain cases. 7-Methyladenine showed no detectable contamination (<2% by silica TLC) by adenine.

Concentrations of the bases were determined spectrophotometrically. Extinction coefficients at neutral pH were taken from the literature (Brown, 1962; Scott, 1964; Lawley, 1971;

Fasman, 1975) or, in a few cases, determined directly by us (units are in $M^{-1}\,cm^{-1}$ in all cases; isocytosine, $\epsilon_{284} = 4{,}500$; 6-methylisocytosine, $\epsilon_{268} = 4{,}800$; 1-methylcytosine $\epsilon_{272} = 8{,}500$; thiocytosine, $\epsilon_{284} = 18{,}000$; 6-thioguanine, $\epsilon_{340} = 24{,}000$; guanosine 2,3-acyclic dialcohol $\epsilon_{251} = 14{,}700$; 7-deazaguanine, $\epsilon_{257} = 10{,}600$).

Buffers used in the kinetic experiments were Tris [tris-(hydroxymethyl) aminomethane] at pH 7.5 and MES [2-(*N*-morpholino)ethanesulphonic acid] at pH 6.5.

Methods

General kinetic methods

The hammerhead construct used in this study, HH16, is kinetically and thermodynamically well characterized (Hertel et al., 1994), which allows the isolation of individual steps of the catalytic process. All reactions were single turnover and were carried out essentially as described (Peracchi et al., 1996). Briefly, substrate (0.1–1 nM) and excess ribozyme (0.6 μM) were heated together for 2 min at 95 °C in 50 mM Tris-HCl, pH 7.5, and subsequently equilibrated 15–30 min at 25 °C to allow annealing, before starting the reaction by the addition of $MgCl_2$ (10 mM final concentration). Control reactions in which the final concentration of ribozyme was varied indicated that the substrate was completely bound in all cases. Aliquots from the reaction mixture were removed at appropriate times and quenched. Products and substrates were separated on 20% polyacrylamide/7 M urea gels, and their ratios at each time point were quantitated using a Molecular Dynamics PhosphorImager. Nonlinear least-squares fits of the data to an exponential function (KaleidaGraph, by Synergy Software, or SigmaPlot, by Jandel Scientific) yielded the observed first-order rate constants for the cleavage of the ribozyme·substrate complex (k_2).

Determination of k_{rescue}.

In base-rescue experiments, the observed rate constant for the cleavage of the ribozyme·substrate complex (k_2^{obs}) was determined in the presence of various concentrations of free base (Peracchi et al., 1996). Values of k_2^{obs} obtained at three- to six-base concentrations were fit by nonlinear least squares to Equation 1 (see Results). This equation was derived from the model in Figure 3 assuming that the chemical step is rate-limiting at all base concentrations. This assumption is supported by control experiments showing that k_2^{obs} at a given [Base] (e.g., k_2^{obs} for A9*X* in the presence of 3 mM adenine or 10 mM purine) is pH-dependent; that is, k_2^{obs} is ~10-fold higher at pH 7.5 than at pH 6.5 (Dahm et al., 1993).

Because plots of k_2^{obs} versus [Base] were nearly linear up to the highest base concentrations used and showed no clear saturation (e.g., see Fig. 8A above), separate K_d and k_2' values could not be determined. However $k_{rescue}(=k_2'/K_d)$, the slope of the linear portion of the dependence, could be readily determined (Peracchi et al., 1996). For each base, values of k_2 and k_{rescue} along with the highest base concentration used allows calculation of the maximum rate enhancement observed in the experiments herein. The rate constants are reported in the tables, and the highest base concentrations used are: 50 mM cytosine, 50 mM isocytosine, 25 mM 6-methyl isocytosine, 21 mM 1-methylcytosine, 11 mM thiocytosine, 30 mM uracil, 11 mM purine (higher concentrations of purine were inhibitory for the wild-type HH16 and were therefore avoided), 5 mM hypoxanthine, 3 mM adenine, 6 mM 1-methyladenine, 0.5 mM 2-methyladenine (at concentrations above 0.1 mM, this base showed apparent saturation with both A9*X* and A13*X*), 23 mM 3-methyladenine, 13 mM 7-methyladenine, 18 mM adenosine, 10 mM 2-aminopurine, 0.03 mM guanine, 1.6 mM 1-methylguanine, 2 mM 3-methylguanine, 0.25 mM 7-methylguanine, 0.35 mM 6-thioguanine, 0.04 mM 8-hydroxyguanine, 0.05 mM 8-mercaptoguanine, 1.5 mM guanosine, 40 mM guanosine acyclic dialcohol, 3 mM 7-deazaguanine, 10 mM caffeine (higher concentrations were inhibitory), 33 mM theophylline, 1.8 mM theobromine, 0.5 mM xanthine, 11 mM indole, and 0.05 mM pterin.

Duplex stability measurements by substrate inhibition

In the substrate inhibition method, a competitive inhibitor is used that binds to the substrate rather than to the enzyme. An oligonucleotide is added that blocks the reaction by forming a duplex with the substrate, and the dissociation constant for the duplex between the inhibitor oligonucleotide and substrate can be determined from the inhibition (Narlikar et al., 1997). Here we used duplexes containing abasic sites to determine whether bases complementary to the residue across from the abasic site can bind to the duplexes. The duplexes were formed between an HH16 substrate (S′ or S'_{C17A}; the difference between the two substrates is the identity of the nucleotide at the cleavage site) with a complementary trap strand (T_X,T'_X or T''_X), as shown in Figure 10A. The experimental strategy is outlined in Figure 10B. The inhibition is a function of the concentration of both the inhibitor oligonucleotide and the exogenous base, as described by Equation 3, which was derived from Figure 10B.

$$k_{obs} = k^0_{obs}/(1 + [Trap]/K_{duplex} + [Trap][Base]/K_{duplex}K_d^{Base}) \quad (3)$$

k^0_{obs} is the rate constant observed in the absence of both trap strand and base, K_{duplex} is the dissociation constant for the duplex between trap and the substrate and K_d^{Base} is the dissociation constant of the base from the abasic duplex.

First the stability of each duplex in Figure 10A was determined in the absence of base, in 50 mM MES-Na, pH 6.5, and 10 mM $MgCl_2$ at 35 °C. The stability of duplex $D1_{AX}$ was also determined in 50 mM Tris-HCl, pH 7.5, and 10 mM $MgCl_2$ at 25 °C. A solution containing 5′-end-labeled substrate and another solution containing the wild-type HH16 and various concentrations of trap strand were heated separately at 95 °C for 1 min in buffer to disrupt potential aggregates and then cooled to room temperature. $MgCl_2$ was added to the ribozyme solution, and the tubes were equilibrated at the reaction temperature for 10 min before starting the reaction by addition of the substrate. The final substrate concentration was ~0.2 nM and the final ribozyme concentration was 8 nM. This ribozyme concentration was well below $K_{1/2}$ (i.e., the

concentration of HH16 needed to reach 50% of the maximum observed rate) as established by control experiments in which the ribozyme concentration was varied. Results from control experiments in which substrate and trap were preequilibrated together before starting the reaction with the addition of ribozyme indicated that establishment of the binding equilibrium is fast relative to cleavage.

In the absence of base, Equation 3 reduces to Equation 4 (Narlikar et al., 1997), which was used to fit the change in the observed cleavage rate constant (k^0_{obs}) at varying trap concentrations.

$$k_{obs} = k^0_{obs}/(1 + [Trap]/K_{duplex}) \quad (4)$$

The values of K_{duplex} obtained at 35 °C (2.5, 9, 2, and 6 μM for $D1_{AX}$, $D1_{GX}$, $D1_{CX}$, and $D2_{UX}$, respectively) will be discussed elsewhere (A. Peracchi, L. Beigelman, A. Karpeisky, L. Maloney and D. Herschlag, in press). The value of K_{duplex} for $D1_{AX}$ at 25 °C was 180 nM.

To determine whether the duplexes were stabilized by exogenous binding of the missing base, activity measurements were carried out in the presence of trap strand at a fixed concentration ~10-fold higher than K_{duplex}. Under these conditions, any additional stabilization of the duplex by the exogenous base would decrease the observed rate of cleavage, as remaining free substrate would be removed from solution, and the dependence of the observed cleavage rate on [Base] is described by Equation 5, which was derived from Equation 3 with [Trap] $\gg$ K_{duplex}.

$$k_{obs} = k^{noBase}_{obs}/(1 + [Base]/K_d^{Base}) \quad (5)$$

The term k^{noBase}_{obs} ($= k^0_{obs} \times K_{duplex}/[Trap]$) is the value of k_{obs} in the presence of trap strand but in the absence of exogenous base.

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