Minimal and extended hammerheads utilize a similar dynamic reaction mechanism for catalysis

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ABSTRACT

Analysis of the catalytic activity of identical mutations in the catalytic cores of nHH8, a very active “extended” hammerhead, and HH16, a less active “minimal” hammerhead, reveal that the tertiary Watson–Crick base pair between C3 and G8 seen in the recent structure of the *Schistosoma mansoni* extended hammerhead can be replaced by other base pairs in both backgrounds. This supports the model that both hammerheads utilize a similar catalytic mechanism but HH16 is slower because it infrequently samples the active conformation. The relative effect of different mutations at positions 3 and 8 also depends on the identity of residue 17 in both nHH8 and HH16. This synergistic effect can best be explained by transient pairing between residues 3 and 17 and 8 and 13, which stabilize an inactive conformation. Thus, mutants of nHH8 and possibly nHH8 itself are also in dynamic equilibrium with an inactive conformation that may resemble the X-ray structure of a minimal hammerhead. Therefore, both minimal and extended hammerhead structures must be considered to fully understand hammerhead catalysis.

Keywords: ribozyme; catalytic RNA; conformational change

INTRODUCTION

The hammerhead ribozyme was originally defined as an RNA self-cleaving motif consisting of three helices intersecting at a catalytic core of 13 single-stranded conserved residues (Buzayan et al. 1986; Hutchins et al. 1986; Forster and Symons 1987a,b; Uhlenbeck 1987). Such a “minimal” hammerhead cleaves efficiently at a unique site at a rate of $\sim 1 \text{ min}^{-1}$ at 25°C in neutral buffers containing 10 mM magnesium ions (Hertel et al. 1994; Stage-Zimmermann and Uhlenbeck 1998). Several X-ray crystal structures of minimal hammerheads show that the three helices adopt an overall Y shape with the adjoining catalytic core consisting of two distinct domains (Pley et al. 1994; Scott et al. 1995, 1996; Murray et al. 2000). However, the structures did not suggest an obvious catalytic mechanism and could not explain a substantial amount of biochemical data on modified hammerheads (McKay 1996; Verma et al. 1997; Blount and Uhlenbeck 2005). It was subsequently found that natural hammerheads contain nonconserved hairpin loops or internal loops within helices I and II that form a

tertiary interaction (De la Pena et al. 2003; Khvorova et al. 2003). Such “extended” hammerheads show rate constants that are at least 100-fold faster than minimal hammerheads under comparable reaction conditions (De la Pena et al. 2003; Khvorova et al. 2003; Canny et al. 2004; Nelson et al. 2005; Roychowdhury-Saha and Burke 2006; Canny et al. 2007). A recent crystal structure of an extended hammerhead from *Schistosoma mansoni* confirmed the presence of the tertiary interaction and revealed a structure of the catalytic core that is substantially different from the arrangement of the core within the minimal hammerhead structures (Martick and Scott 2006). Instead of folding into the two separate domains present in the minimal hammerhead structure, the entire catalytic core of the extended hammerhead folds into a single unit with the scissile phosphodiester bond lying adjacent to potential catalytic residues. In addition, although activity in the X-ray structure is blocked by the presence of a 2'-O-methyl group, the positions of the 2' oxygen, the scissile phosphate, and the 5' oxygen are nearly aligned for the expected in-line attack. Thus, the extended hammerhead has a structure that seems close to the known transition state required by the $S_N2(P)$ mechanism (van Tol et al. 1990; Koizumi and Ohtsuka 1991; Slim and Gait 1991), providing a convincing rationale for the faster observed rate.

Despite lacking the rate enhancing tertiary interaction and possessing a quite different core structure, the minimal

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hammerhead is actually a rather effective ribozyme, cleaving at a rate of $\sim 10^6$ -fold faster than the average uncatalyzed rate of phosphodiester bond cleavage (Hertel et al. 1997; Li and Breaker 1999; Soukup and Breaker 1999). Since the extended hammerheads cleave only several hundred-fold faster, it is clear that the minimal hammerhead still possesses at least two thirds of the catalytic rate enhancement associated with hammerhead catalysis. Two possible explanations for the activity of the minimal hammerhead have been proposed. In the first, the minimal hammerhead utilizes a mechanism involving relatively small structural rearrangements around the cleavage site to achieve a configuration appropriate for in-line attack. This suggestion, originally made by Pley et al. (1994), was supported by a series of structures of derivatives of a minimal hammerhead, including ones with and without bound magnesium ions, a talo-5'-C-methyl modification at the cleavage site, and a product complex (Scott et al. 1996; Murray et al. 1998, 2000). By ordering these structures in progressively greater in-line arrangement about the scissile phosphate, the potential reaction path earlier proposed was supported (Scott et al. 1996; Murray et al. 1998). A second possibility, suggested by Peracchi et al. (1997, 1998), is that the core of the minimal hammerhead exists in multiple conformations and only infrequently adopts an active conformation that is substantially different from the minimal structure. Subsequent cadmium ion "rescue" experiments of minimal hammerheads containing phosphorothioates (Wang et al. 1999) suggested that the transition state structure involved close approach of phosphates 9 and 1.1, which are distant in the minimal structures. The juxtaposition of these two phosphates observed in the *Schistosoma* structure clearly supports this isomerization model for the minimal hammerhead, but additional biochemical experiments are needed to confirm it.

In this article, we compare the effect on catalysis for an identical set of mutations made in the catalytic cores of well characterized minimal and extended hammerheads. The mutations were designed to test the importance of the tertiary Watson–Crick base pair between C3 and G8 that is present in the extended, but not in the minimal, hammerhead structure. In addition, position 17 was mutated, since it interacts with C3 in the minimal hammerhead structure. If both hammerheads show a similar requirement for the C3-G8 base pair, the data would imply that they have a similar transition state structure. This would support the model that the minimal hammerhead rapidly samples multiple conformations and is only catalytically active when it adopts a conformation that resembles the *Schistosoma* core structure.

RESULTS

Mutagenesis focused on three positions within the core of the hammerhead ribozyme: the completely conserved

residues C3 and G8 and the more variable cleavage site nucleotide, N17, which is commonly a C or an A, occasionally a U, and never a G (Garrett et al. 1996; Flores et al. 2001; Carbonell et al. 2006). In the extended hammerhead structure (Fig. 1A), C3 and G8 form a Watson–Crick tertiary base pair that stacks between the terminal G2.1-C1.1 base pair of helix I and uridine 7, while C17 is near G12, A13, and A14 (Martick and Scott 2006). In the minimal hammerhead structure (Fig. 1C), G8 forms a sheared base pair with A13 and both stack between the G12-A9 and A14-U7 pairs within domain 2, while C3 forms a single hydrogen bond with C17 and the two residues stack between the G2.1-C1.1 pair and uridine 4 in domain 1. Previous mutagenesis experiments in minimal hammerheads established that point mutations of either C3 or G8 greatly reduced cleavage, and double mutants between these positions were never tested (Ruffner et al. 1990; Tuschl et al. 1993; Murray et al. 1995; Peracchi et al. 1996; Kore et al. 2000). Mutations of position 17 made in several minimal hammerheads were active in the order C17 > A17 > U17 (Koizumi et al. 1988a; Baidya and Uhlenbeck 1997), while G17 was inactive unless additional mutations were made elsewhere in the catalytic core (Koizumi and Ohtsuka 1991; Vaish et al. 1998; Kore et al. 2000; Eckstein et al. 2001). Experiments with double mutants suggest that strong N3-N17 pairs were deleterious to cleavage (Koizumi and Ohtsuka 1991; Baidya et al. 1997; Baidya and Uhlenbeck 1997). A limited mutagenesis of the C3-G8 pair in the *Schistosoma* hammerhead revealed that while the G3 single mutant showed dramatically reduced cleavage, the G3C8 double mutation partially restored activity, supporting the new structure (Martick and Scott 2006). Mutagenesis of C3 and G8 to other base pairs in the *Arabidopsis* and PLMVd hammerheads have recently also confirmed the presence of this base pair (Przybilski and Hammann 2007).

Two kinetically well-characterized hammerheads were chosen for mutagenesis studies. The minimal HH16 (Fig. 1D) has previously been studied extensively (Hertel et al. 1994), while the extended nHH8 (Fig. 1B) is a derivative of the hammerhead in the plus strand of the satellite of lucerne transient streak virus [sLTSV(+)], which contains a lengthened helix III to permit stable binding. Since hammerhead catalysis involves the transesterification of an internucleotide phosphodiester bond to a 2', 3' cyclic phosphate product, the reaction is fully reversible and is expected to show the same apparent rate constant (k_{obs}) and reach the same fraction of uncleaved molecules at equilibrium (f_{eq}) when the assay is performed using the forward, cleavage reaction or the reverse, ligation reaction. HH16 is usually assayed in a cleavage reaction where the ribozyme and substrate strands are annealed and the reaction is started by addition of MgCl_2 . At 10 mM MgCl_2 (pH 7.5), cleavage kinetics of HH16 follows a single exponential with a $k_{\text{obs}} = 0.7 \text{ min}^{-1}$ and $f_{\text{eq}} = 0.15$ (Hertel

FIGURE 1. The tertiary and secondary structures of extended and minimal hammerheads. Critical core nucleotides include: C17 (purple), C3 (green), G8 (blue), and A13 (orange). (A) The four nucleotides as they are arranged in the crystal structure of the catalytic core of a noncleavable extended hammerhead from *Schistosoma* (PDB 2GOZ) (Martick and Scott 2006). (B) Secondary structure of nHH8 derived from sLTSV(+) with the core arranged to reflect the crystal structure. (C) The four nucleotides arranged in the catalytic core of a noncleavable minimal hammerhead (PDB 1HMH) (Pley et al. 1994). (D) The secondary structure of the minimal HH16 (Hertel et al. 1994) with a core arranged in the two distinct domains seen in the X-ray structure.

et al. 1994). When HH16 is assayed in a ligation reaction under the same conditions, $k_{\text{obs}} = 0.95 \text{ min}^{-1}$ and $f_{\text{eq}} = 0.008$ (Hertel et al. 1994). Thus, although the two assays give similar k_{obs} values as expected, the disparity in the f_{eq} data suggests that one of the reactions may contain a fraction of catalytically inactive molecules. Since f_{eq} in the cleavage reaction can be reduced to less than 0.1 by treatment of HH16 with chaperones (Herschlag et al. 1994), it appears that a fraction of the uncleaved HH16 molecules are in a misfolded, inactive conformation that does not become active during the time course of the cleavage reaction.

The activity of nHH8 is generally assayed in a ligation reaction by annealing the two product fragments and starting the reaction by the addition of MgCl_2 (Nelson et al. 2005). At 1 mM MgCl_2 (pH 6.0), a single exponential rate of ligation is observed with a $k_{\text{obs}} = 2.5 \text{ min}^{-1}$ and $f_{\text{eq}} = 0.052$ (Table 1). In 10 mM MgCl_2 (pH 7.5), k_{obs} is a much faster 470 min^{-1} (I. Shepotinovskaya, unpubl.). Determining k_{obs} for nHH8 using a cleavage assay is technically challenging because the full-length hammerhead must be purified from a transcription reaction containing an inhibitory complementary DNA fragment and then re-annealed in a buffer without MgCl_2 , a process that often yields partially cleaved molecules (Nelson et al. 2005). Cleavage reactions are then initiated by the addition of MgCl_2 . At 1 mM MgCl_2 (pH 6.0), the cleavage assay gave values of $k_{\text{obs}} = 3.2 \text{ min}^{-1}$ and $f_{\text{eq}} = 0.05$ for nHH8 (I. Shepotinovskaya, unpubl.). While the cleavage data are less reliable, the close agreement of both k_{obs} and f_{eq} in the two assays suggests that, unlike HH16, nHH8 contains relatively few inactive molecules.

The activity of nHH8 and the G3, C8, G3C8, A3U8, and U3A8 mutations was determined in ligation reactions where the 51-nucleotide (nt) ribozymes were each combined with a $[5'\text{-P}^{32}]$ -labeled 9-nt product containing C17 and the rate determined in 1 mM MgCl_2 (pH 6.0). An individual set of rate determinations is shown in Figure 2A. For the wild-type C3G8 and the three other N3N8 mutants, values of f_{eq} and k_{obs} could be obtained by fitting the data to a single exponential. Average f_{eq} and k_{obs} values from multiple determinations are summarized in Table 1.

The G3 and C8 single mutations ligated so slowly that it was not possible to approach completion in the 1 mM MgCl_2 (pH 6.0) buffer even after several days of incubation. Instead, reactions were performed at 10 mM MgCl_2 (pH 7.5), where the reaction rate was fast enough that values of f_{eq} could be extrapolated from data collected over 30 h and k_{obs} values calculated (Fig. 2A, inset). If it is assumed that the two point mutations show a similar dependence of k_{obs} on pH and magnesium ion concentration as wild type, these values of k_{obs} should be divided by a factor of 3200 to estimate the cleavage rate in the 1 mM MgCl_2 (pH 6.0)

TABLE 1. Ligation data for nHH8

	f_{eq}	k_{obs} (min ⁻¹)	k_{rel} ^a
C17 hammerheads			
C3/G8	0.052 ± 0.01	2.5 ± 1.1	1.0
G3/C8	0.16 ± 0.06	0.017 ± 0.005	6.8 × 10 ⁻³
A3/U8	0.026 ± 0.009	2.7 × 10 ⁻³ ± 1.6 × 10 ⁻³	8.8 × 10 ⁻⁴
U3/A8	0.069 ± 0.014	0.030 ± 0.009	1.2 × 10 ⁻²
A17 hammerheads			
C3/G8	0.045 ± 0.018	0.95 ± 0.52	1.0
G3/C8	9.6 × 10 ⁻³ ± 2.5 × 10 ⁻³	0.063 ± 0.017	5.2 × 10 ⁻²
A3/U8	0.046 ± 0.021	4.0 × 10 ⁻⁴ ± 1.6 × 10 ⁻⁴	4.1 × 10 ⁻⁴
U3/A8	0.33 ± 0.06	1.6 × 10 ⁻³ ± 2.0 × 10 ⁻⁴	1.3 × 10 ⁻³
U17 hammerheads			
C3/G8	0.14 ± 0.02	0.046 ± 0.014	1.0
G3/C8	0.073 ± 0.032	2.5 × 10 ⁻³ ± 1.7 × 10 ⁻³	5.4 × 10 ⁻²
A3/U8	0.029 ± 0.0060	9.4 × 10 ⁻⁵ ± 3.5 × 10 ⁻⁶	2.0 × 10 ⁻³
U3/A8	0.30 ± 0.07	2.8 × 10 ⁻⁴ ± 9.1 × 10 ⁻⁵	4.8 × 10 ⁻³
G17 hammerheads			
C3/G8	0.042 ± 0.016	0.78 ± 0.30	1.0
G3/C8	0.027 ± 0.008	0.012 ± 0.0046	1.5 × 10 ⁻²
A3/U8	n.d.	n.d.	—
U3/A8	0.28 ± 0.07	2.3 × 10 ⁻³ ± 1.0 × 10 ⁻³	2.9 × 10 ⁻³

Data collected in 50 mM Mes (pH 6.0), 1 mM MgCl₂, at 25°C using the ligation assay. Each value was the average (± SD) of at least three independent determinations like those shown in Figure 2A. n.d. indicates that although tested, very low activity prevented quantitation. ^a k_{rel} is the value of k_{obs} for a given 3–8 mutation divided by the wild-type k_{obs} in a given N17 background.

buffer (see Materials and Methods). These calculated values are $k_{obs} = 8.2 \times 10^{-7} \text{ min}^{-1}$ for the C8 mutations and $k_{obs} = 1.6 \times 10^{-6} \text{ min}^{-1}$ for the G8 mutations, but as extrapolations, the values should only be considered estimates.

In agreement with similar experiments done on the *Schistosoma* hammerhead (Martick and Scott 2006) and the *Arabidopsis* and PLMVd hammerheads (Przybilski and Hammann 2007), the experiments on nHH8 confirm the presence of the C3–G8 tertiary base pair seen in the *Schistosoma* crystal structure. The two point mutants G3 and C8 reduce k_{obs} by more than six orders of magnitude, which is consistent with the importance of this pair in the folded structure. The double mutant G3C8, which should also be capable of forming a base pair, shows a k_{obs} 10,000-fold faster than the fastest single mutant but remains 150-fold slower than nHH8. These results are quantitatively similar for the identical mutations in the *Schistosoma* hammerhead, where a $k_{obs} = 50 \text{ min}^{-1}$ for the native hammerhead (Canny et al. 2004) is reduced by ~500,000-fold for the single C8 mutant and 230-fold for the G3C8 double mutant (Martick and Scott 2006). Finally, the A3U8 and U3A8 double mutants of nHH8 both show faster k_{obs} than the G3 or C8 single mutants, suggesting that they also form a tertiary base pair. Interestingly, the U3A8 derivative has a slightly faster k_{obs} than the potentially more stable G3C8 mutant. However, it is striking that none of the double mutants come close to restoring the activity to that of the wild-type C3–G8 pair.

Similar to what has been observed for the *Schistosoma* hammerhead (Canny et al. 2007) and another extended hammerhead (Nelson et al. 2005), the value of $f_{eq} = 0.052$ determined by the ligation reaction is significantly greater than the value of $f_{eq} = 0.008$ for the minimal HH16 ligation reaction. An interesting feature of the data in Figure 2A is that each N3–N8 pair shows a different f_{eq} , including a $f_{eq} = 0.16$ for the G3C8 mutation that is greater than wild type and a $f_{eq} = 0.026$ for the A3U8 mutations that is much less than wild type. These differences do not correlate with the value of k_{obs} and thus reflect some independent property of each mutation.

The same series of five nHH8 mutations at positions 3 and 8 were then assayed with substrates containing A17, U17, or G17 in a manner similar to Figure 2A. In each case, k_{obs} of the C3 and G8 single mutations was very slow, permitting only estimates of k_{obs} in the range of $6 \times 10^{-7} - 3 \times 10^{-8}$ using data obtained at 10 mM MgCl₂ (pH 7.5) as discussed above. Table 1 reports average k_{obs} and f_{eq} values for the four N3–N8 pairs with different residues at N17. It is clear that the identity of N17 affects both k_{obs} and f_{eq} in nHH8. The wild-type C17 hammerhead is two- to threefold faster than the A17 and G17 derivatives and 50-fold faster than the U17 derivative. In contrast, f_{eq} is greatest in the U17 derivative and smallest in the G17 derivative.

The effect of the different mutations at positions 3 and 8 clearly varies with the identity of N17. This is most easily seen by comparing the relative rate constant (k_{rel}) of each three to eight mutation with that of the C3G8 wild type in each group of N17 hammerheads (Table 1). The values of k_{rel} for the three 3–8 base pairs (bp) differ significantly in each N17 set. The most striking effect is that all three of the other N17 hammerheads are much more able to tolerate the G3C8 double mutation than the C17 hammerhead. Thus, in contrast to the 150-fold decrease in k_{obs} determined with the C17 hammerhead; the U17 hammerhead containing the G3C8 inversion shows a k_{obs} that is only 20-fold slower than wild type. Other clear differences in k_{rel} include the very poor activity of U3A8 mutation in the A17 hammerhead and the somewhat faster k_{obs} of the A3U8 mutation in the U17 hammerhead. The different combinations of N17 with N3–N8 pairs also show a wide range of f_{eq} values that do not correlate with either sequence or k_{obs} value. For example, the G3C8 mutant gives a greater f_{eq} than wild type with C17, but it is less with A17 even though the k_{rel} for A17 is greater.

FIGURE 2. Rate of approach to equilibrium of nHH8 and HH16 and their G3, C8, G3C8, A3U8, U3A8 mutations. (A) Single turnover bimolecular ligation reactions for nHH8 at 1 μ M ribozyme and trace substrate concentrations at 25°C in 50 mM Mes (pH 6.0), 1 mM MgCl₂ (solid symbols), or 50 mM Hepes (pH 7.5), 10 mM MgCl₂ (inset open symbols). Each experiment was fit to a single exponential with a k_{obs} and f_{eq} , the fraction of full-length product at the end of the reaction. C3G8 wild type (●) $k_{obs} = 3.2 \text{ min}^{-1}$, $f_{eq} = 0.061$; G3C8 (■) $k_{obs} = 0.012 \text{ min}^{-1}$, $f_{eq} = 0.20$; A3U8 (◆) $k_{obs} = 0.0037 \text{ min}^{-1}$, $f_{eq} = 0.018$; U3A8 (▼) $k_{obs} = 0.030 \text{ min}^{-1}$, $f_{eq} = 0.079$; C8 (□) $k_{obs} = 0.0012 \text{ min}^{-1}$, $f_{eq} = 0.45$; and G3 (○) $k_{obs} = 0.0015 \text{ min}^{-1}$, $k_{obs} = 0.0021 \text{ min}^{-1}$ in the inset. (B) Single turnover bimolecular cleavage reactions for minimal HH16 at 25°C in 100 mM Hepes (pH 7.5), 10 mM MgCl₂ (solid symbols), or 100 mM Tris-HCl (pH 8.1), 100 mM MgCl₂ (open symbols). Experiments fit to a single exponential with k_{obs} and f_{eq} , the fraction of full-length product at the end of the reaction: C3G8 wild type (●) $k_{obs} = 0.35 \text{ min}^{-1}$, $f_{eq} = 0.32$; G3C8 (■) $k_{obs} = 0.00037 \text{ min}^{-1}$, $f_{eq} = 0.3$; A3U8 (◆) $k_{obs} = 0.00083 \text{ min}^{-1}$, $f_{eq} = 0.18$; U3A8 (▼) $k_{obs} = 0.0029 \text{ min}^{-1}$, $f_{eq} = 0.13$; G3 (○) $k_{obs} = 3.7 \times 10^{-6} \text{ min}^{-1}$, $f_{eq} = 0.3$; and C8 (□) $k_{obs} = 7.4 \times 10^{-6} \text{ min}^{-1}$, $f_{eq} = 0.3$.

In order to test whether a minimal hammerhead also shows evidence for a tertiary 3–8 bp, wild-type HH16 and the G3, C8, G3C8, A3U8, and U3A8 mutant ribozymes were assayed in cleavage reactions with the four different

[5'-P³²]-labeled, 17-nt substrates containing each N17. Cleavage reactions were chosen instead of ligation reactions in this case because the extent of ligation is too low to permit accurate determination of the slow k_{obs} values. Figure 2B shows a single data set for the C17 substrate in 10 mM MgCl₂ (pH 7.5). For the wild-type C3G8 hammerhead, rapid cleavage ($k_{obs} = 0.50$) to a $f_{eq} = 0.39$ was followed by a much slower component, consistent with very slow conversion of a fraction of inactive molecules to an active state. This confirms previous experiments showing that HH16 has a fraction of inactive species (Herschlag et al. 1994; Hertel et al. 1994), although the very slow second phase of the reaction had not been reported. Under these conditions, nearly complete progress curves could be obtained for the A3U8 and U3A8 hammerheads after 3000 min, but the G3C8 required > 5000 min to approach completion. Averages of multiple determinations of k_{obs} and f_{eq} values for the four N3N8 mutations are summarized in Table 2.

For the G3 and C8 single mutations, cleavage was very slow and was incomplete even in 100 mM MgCl₂ (pH 8.1) (Fig. 2B, inset). To estimate k_{obs} values that could be compared with the other data, an $f_{eq} = 0.3$ was assumed and the rates were divided by 40 to account for the higher pH and MgCl₂ concentrations (see Materials and Methods). These estimated values were $k_{obs} = 1.7 \times 10^{-7} \text{ min}^{-1}$ for the C8 mutant and $k_{obs} = 4.2 \times 10^{-7} \text{ min}^{-1}$ for the G3 mutant, which are both similar to estimates of the uncatalyzed rate of RNA cleavage in the same buffer (Hertel et al. 1997; Li and Breaker 1999; Soukup and Breaker 1999).

Corresponding cleavage data for the four N3-N8 derivatives of HH16 were also collected for the A17, U17, and G17 substrates (Table 2). In the case of the A17 and U17 substrates, the much slower cleavage of the N3-N8 mutants made it necessary to estimate an f_{eq} value to calculate k_{obs} . While these estimates were consistent with values obtained upon very long (10–14 d) incubations (see Materials and Methods), this reduces the accuracy of k_{obs} and, given that native HH16 contains a significant fraction of inactive species, also prevents interpretation of f_{eq} data for the different HH16 mutants.

As had been found with other minimal hammerheads (Koizumi et al. 1988b; Ruffner et al. 1990; Koizumi and Ohtsuka 1991; Baidya and Uhlenbeck 1997), the G17 substrates cleaved very poorly even after very long incubation times. However, based upon the relatively robust reactivity of G17 derivatives of nHH8, we re-evaluated the data and found that HH16(G17) shows biphasic kinetics, with ~4% of the molecules cleaving with a $k_{obs} = 0.42 \text{ min}^{-1}$ (pH 7.5) at 10 mM MgCl₂ (Fig. 3A). The remaining molecules cleaved site-specifically but so slowly that the reaction was incomplete after 7000 min (Fig. 3A, inset). This suggests that the majority of the HH16(G17) molecules were trapped in an inactive structure that is very slowly converted to the active structure. In support of this

TABLE 2. Cleavage data for HH16

	f_{eq}	k_{obs} (min ⁻¹)	k_{rel} ^a
C17 hammerheads			
C3/G8	0.39 ± 0.08	0.50 ± 0.16	1.0
G3/C8	0.26 ± 0.20	$3.8 \times 10^{-4} \pm 1.7 \times 10^{-4}$	7.6×10^{-4}
A3/U8	0.16 ± 0.03	$7.9 \times 10^{-4} \pm 7.7 \times 10^{-5}$	1.6×10^{-3}
U3/A8	0.14 ± 0.02	$3.0 \times 10^{-3} \pm 9.0 \times 10^{-4}$	6.0×10^{-3}
A17 hammerheads			
C3/G8	0.45 ± 0.07	0.35 ± 0.15	1.0
G3/C8	0.24 ± 0.10	$1.4 \times 10^{-3} \pm 4.0 \times 10^{-4}$	4.0×10^{-3}
A3/U8	[0.30]	$3.5 \times 10^{-4} \pm 1.6 \times 10^{-4}$	1.0×10^{-3}
U3/A8	[0.30]	$2.0 \times 10^{-4} \pm 9.7 \times 10^{-5}$	5.7×10^{-4}
U17 hammerheads			
C3/G8	0.48 ± 0.03	0.023 ± 0.011	1.0
G3/C8	[0.30]	$2.5 \times 10^{-4} \pm 1.8 \times 10^{-5}$	1.1×10^{-2}
A3/U8	[0.30]	$5.8 \times 10^{-5} \pm 3.4 \times 10^{-5}$	2.5×10^{-3}
U3/A8	[0.30]	$2.8 \times 10^{-4} \pm 3.4 \times 10^{-5}$	1.2×10^{-2}
G17 hammerheads			
C3/G8	0.97 ± 0.04	0.42 ± 0.26	1.0
G3/C8	0.94 ± 0.016	$8.7 \times 10^{-4} \pm 4.0 \times 10^{-4}$	2.1×10^{-3}
A3/U8	0.95 ± 0.010	$1.9 \times 10^{-4} \pm 7.4 \times 10^{-5}$	4.5×10^{-4}
U3/A8	n.d.	n.d.	—

Data collected at 100 mM Hepes (pH 7.5), 10 mM MgCl₂, at 25°C using the cleavage assay. Each value is the average (±SD) of at least three independent determinations like those shown in Figure 2B. n.d. indicates that although tested, very low activity prevented quantitation. Bracketed values indicate f_{eq} value assumed to fit data for k_{obs} .

^a k_{rel} is the value of k_{obs} for a given three to eight mutation divided by the wild-type k_{obs} in a given N17 background.

model, the log of the rate of the fast cleavage component was proportional to pH as expected for hammerhead cleavage, while the rate of slow component was only slightly affected by pH, more consistent with a conformational change. Furthermore, when HH16(G17) was subjected to multiple rounds of temperature cycling, the extent of cleavage could be increased, which is consistent with a model that a fraction of the inactive complexes becomes active upon incubation at 65°C (Fig. 3B). Thus, contrary to previous conclusions, it appears that minimal hammerheads containing G17 are quite active but are primarily trapped in an inactive conformation that only exchanges with the active conformation very slowly. Thus, the k_{obs} data for the G17 derivatives summarized in Table 2 report only the early phase of the reaction.

The data in Figure 2B and Table 2 clearly establish that a 3–8 bp is also required for HH16 activity. As has previously been observed with other minimal hammerheads (Ruffner et al. 1990; Tuschl et al. 1993; Murray et al. 1995; Peracchi et al. 1996; Kore et al. 2000), both the G3 and C8 point mutations reduce k_{obs} to a value that approaches the rate for noncatalyzed cleavage of RNA. However all three HH16 mutants that form a base pair between positions 3 and 8 cleave from 34- to 18,000-fold faster than the point mutations but cleave much slower than the wild-type C3-G8 pair. Also similar to nHH8, the ability of the different 3–8 bp to support HH16 cleavage depends upon the

identity of N17. For example, the G3-C8 pair is much more effective at replacing the C3-G8 pair with U17 than with C17 (Table 2).

In general, the response of HH16 and nHH8 to the same set of mutations is strikingly similar. Figure 4 correlates k_{obs} in the two hammerhead backgrounds for the 14 mutations from Tables 1 and 2 where accurate k_{obs} values are available in both cases. Although the different mutations reduce k_{obs} of each hammerhead by up to four orders of magnitude, their effects are quite comparable in each background. While the two sets of data were obtained under different buffer conditions for experimental convenience, it is unlikely that this will strongly effect the conclusion. To confirm this, k_{obs} for seven mutations in each hammerhead was measured in the same buffer (10 mM MgCl₂ at pH 7.5), and k_{obs} for a mutation in nHH8 was consistently faster (35- to 1400-fold) than it was for HH16 (data not shown). The similar effect of different mutations on k_{obs} in two hammerheads strongly supports the

idea that both ribozymes proceed through a similar transition state.

DISCUSSION

Mutagenesis experiments establish that the C3-G8 tertiary base pair seen in the crystal structure of the *Schistosoma* hammerhead is critical for the fast rate of ligation observed in another extended hammerhead derived from sLTSV(+). The C3 and G8 point mutations that disrupt the pair reduce k_{obs} six to eight orders of magnitude, consistent with a critical role of the pair for maintaining the folded structure of the hammerhead core. However, nHH8 containing the G3C8 double mutant shows a k_{obs} considerably faster than the single mutants and, depending on the identity of the residue at position 17, can have k_{obs} values within 20-fold of the native C3G8 hammerhead. A3U8 and U3A8 substitutions are also partially active. In agreement with the *Schistosoma* structure, the role of this tertiary base pair appears to be structural, so other isosteric base pairs can be successfully substituted without eliminating catalytic function.

An important conclusion of this article is that the C3G8 replacement experiments were equally successful in HH16, a slow cleaving “minimal” hammerhead that lacks any tertiary interaction between helices I and II. The C3 and G8 point mutations of HH16 reduce k_{obs} to a value that approaches

FIGURE 3. Biphasic cleavage kinetics of HH16(G17). (A) Average values determined from at least two independent experiments for initial phase of cleavage at 25°C in 10 mM $MgCl_2$ and (●) 100 mM Hepes (pH 7.5; $k_{obs} = 0.42 \pm 0.26 \text{ min}^{-1}$, $f_{eq} = 0.97 \pm 0.04$); (■) 100 mM Tris HCl (pH 8.1; $k_{obs} = 1.6 \pm 0.4 \text{ min}^{-1}$, $f_{eq} = 0.96 \pm 0.01$); and (◆) 100 mM Mes (pH 6.5; $k_{obs} = 0.031 \pm 0.003 \text{ min}^{-1}$, $f_{eq} = 0.97 \pm 0.01$). Second slow phase of cleavage is shown in the inset. (B) f_{eq} obtained after successive cycles of heating to 65°C and cooling to 25°C shown in gray bars; black bar is control that was not heated to 65°C but held at 25°C for duration with aliquots removed at the end of cycles marked.

the rate of uncatalyzed cleavage of RNA. Versions of HH16 containing the G3C8 double mutant cleave > 1000-fold faster than either of the single mutants and, depending on the identity of the residue at position 17, can have k_{obs} values within 90-fold of the native HH16. A3U8 and U3A8 substitutions can also partially support cleavage. This experiment strongly supports the notion that the C3-G8 pair also forms when HH16 adopts a catalytically active

conformation that presumably resembles the structure of the catalytic core of the *Schistosoma* hammerhead.

NMR experiments performed on several minimal hammerheads indicate that while their catalytic cores are somewhat disordered, they clearly contain the sheared G8-A13 and A9-G12 base pairs that are part of domain 2 of the X-ray structure of the minimal hammerhead (Heus and Pardi 1991; Amano 2006). Since we have shown here that C3 must pair with G8 to form the active conformation, it is clear that the majority of minimal hammerheads in solution are not in an active conformation. This affirms the proposal of Peracchi et al. (1997, 1998) that minimal hammerheads in solution are primarily in inactive conformations that occasionally adopt active conformations. Minimal hammerhead cleavage and ligation can thus be described by three equilibria: (1) the rapid isomerization between the multiple inactive conformations and the single active conformation of the uncleaved hammerhead described by the equilibrium constant K_u ; (2) catalysis of the active hammerhead described by the “internal” equilibrium K_{int} that is the ratio of the elemental forward cleavage (k_2) and reverse ligation (k_{-2}) rate constants; and (3) the rapid isomerization between the single active conformation of the cleaved hammerhead and the multiple inactive conformations described by the equilibrium constant K_c (Fig. 5). The rates of exchange between active and inactive conformations associated with K_u and K_c are very fast and therefore undetectable by experiments that measure the comparatively slow rate of hammerhead catalysis. Intramolecular RNA isomerizations of comparable

FIGURE 4. Correlation between k_{obs} of mutations in minimal and extended hammerheads. k_{obs} for HH16 mutations measured in 10 mM $MgCl_2$, 100 mM Hepes (pH 7.5) at 25°C (Table 2) compared with k_{obs} for same mutations in nHH8 measured in 1 mM $MgCl_2$, 50 mM Mes (pH 6) (Table 1). The best-fit least-squares line has a slope of 0.92.

FIGURE 5. A model for the dynamic cleavage-ligation equilibria of minimal hammerheads. The majority of the uncleaved hammerheads consist of a dynamic mixture of structures (A) that resemble the minimal hammerhead X-ray structure. A small fraction of the uncleaved molecules defined by K_u transiently adopts an active conformation (B) that resembles the *Schistosoma* core structure. The “internal” cleavage-ligation equilibrium (K_{int}) is described the cleavage (k_2) and ligation (k_{-2}) rate constants. Finally, the cleaved active structure (C) is in an equilibrium defined by K_c with a mixture of cleaved inactive structures (D) that also resemble the minimal hammerhead.

complexity in the hairpin ribozyme and the tetraloop–receptor complex are in the low millisecond time scale (Hodak et al. 2005; Wilson et al. 2005; Liu et al. 2007). For this description of the hammerhead reaction, the value of k_{obs} can be expressed by

$$k_{obs} = \left(\frac{K_u}{1 + K_u} \right) k_2 + \left(\frac{1}{1 + K_c} \right) k_{-2},$$

and the value of f_{eq} can be expressed by

$$f_{eq} = \frac{\left(\frac{1}{1 + K_c} \right) k_{-2}}{k_{obs}}.$$

In the absence of independent measurements of K_u and K_c , it is not possible to estimate their values unless additional assumptions are made. If we assume that the $k_{obs} = 470 \text{ min}^{-1}$ and $f_{eq} = 0.05$ determined for nHH8 in 10 mM MgCl_2 (pH 7.5) (I. Shepotinovskaya, unpubl.) reflects an active hammerhead in both the uncleaved and cleaved forms ($K_u \gg 1$, $K_c \ll 1$), then $k_{obs} = k_2 + k_{-2}$ and $f_{eq} = k_{-2}/(k_2 + k_{-2})$. This gives $k_2 = 477 \text{ min}^{-1}$ and $k_{-2} = 23 \text{ min}^{-1}$. Since HH16 shows $k_{obs} = 0.95 \text{ min}^{-1}$ and $f_{eq} = 0.008$ in the same buffer, we can calculate $K_u = 0.002$ and $K_c = 3000$. In other words, the uncleaved HH16 only adopts the active conformation $\sim 0.2\%$ of the time and, once cleaved, is only in the active conformation $\sim 0.03\%$ of the time. The fact that $1/K_c$ is smaller than K_u reflects the fact that the $f_{eq} = 0.008$ for HH16 is less than the $f_{eq} = 0.05$ for nHH8 and indicates that the cleaved form of HH16 adopts a larger fraction of inactive conformations than the uncleaved form. This is consistent with the idea that the cleaved hammerhead is more flexible than the uncleaved because of the break in the phosphodiester bond. A similar conclusion was reached by comparing the properties of hammerheads containing a crosslink between helices 1 and 2 (Stage-Zimmermann and Uhlenbeck 2001; Blount and Uhlenbeck 2002). However, it is important to point out that the assumption that both the cleaved and uncleaved forms of

nHH8 are in a fully active conformation may not be correct. In addition, although the cores of nHH8 and HH16 are identical, subtle differences in structures of the helices could lead to different k_2 or k_{-2} values. If either of these possibilities are the case, the fractions of active HH16 molecules estimated above would be in error.

Since the rapid conformational isomerizations are such an important component in describing HH16 cleavage, they are likely to also contribute to the variability in k_{obs} for the HH16 mutations in Table 2. In all minimal hammerhead crystal structures, G8 pairs with A13, and in several of them, C3 pairs with C17 (Pley et al. 1994; Scott et al. 1995, 1996; Murray et al. 2000). Since these structures represent inactive conformations, mutations that stabilize these pairs would reduce HH16 activity while mutations that destabilize these pairs could increase HH16 activity. Thus, the poor activity for all A3U8 derivatives of HH16 may reflect the fact that a U8-A13 pair is more effective than the native G8-A13 pair in stabilizing domain II and thus maintaining the inactive conformation and decreasing K_u even further. Similarly, as has previously been discussed (Baidya and Uhlenbeck 1997; Simorre et al. 1998), the slower k_{obs} for the G17 and U17 derivatives of HH16 may partially reflect their more stable pairing with C3 than with C17 or A17. Since we now know that C3 must pair with G8 for activity, any interaction that sequesters C3 would reduce cleavage.

The observed “synergistic” dependence of the activity of different 3–8 pairs upon the identity of position 17 in HH16 probably also reflects the relative stabilities of inactive and active conformations. Thus, the reason why the G3C8 inversion mutant has relatively low k_{obs} when C17 is present is because the transient formation of a stable G3-C17 pair effectively competes with the formation of the G3-C8 pair. The G3C8 inversion mutant is relatively more active when either U17, A17, or G17 is present, since the alternative G3-U17, G3-A17, or G3-G17 pairs are weaker and thus do not compete as well. Similarly, the exceptionally poor activity of HH16 containing A3U8 U17 may be the result of two alternative base pairs, A3-U17 and U8-A13, which prevent formation of the A3-U8 pair. In other words, to properly interpret the effect of a HH16 mutation on k_{obs} , it is critical to consider its effect on the conformational isomerizations as well as the cleavage step. Since the structures of minimal hammerheads can be considered to be representative of a set of inactive conformations and the catalytic core of the *Schistosoma* hammerhead is representative of a more active conformation, it is clear that both structures are critical to our understanding of minimal hammerhead function.

It is striking that the differences in k_{obs} for different N17 mutations and the synergies between N17 and the identity of the 3–8 bp are also observed for nHH8. Indeed, as shown in Figure 4, the magnitude of the effects of the mutations in nHH8 is quite similar to those seen in HH16. These results cannot be easily explained solely in terms of

the *Schistosoma* crystal structure. While the somewhat slower k_{obs} for the A17 and G17 versions of nHH8 could potentially be explained by somewhat worse fit of the purine rings into the position of C17, the very poor cleavage of nHH8(U17) is hard to understand since U17 could also make the single hydrogen bond between its two oxygen and the amino group of A13 that is also observed for C17. The *Schistosoma* structure also does not easily explain how the identity of N17 would influence the effect of different 3–8 bp substitutions since N17 is 11–20 Å away from the 3–8 bp.

A better way to rationalize the varying effects of 3–8 bp substitution in different N17 backgrounds for nHH8 is to propose that many of the nHH8 mutations are also in rapid exchange with inactive conformations that resemble the minimal hammerhead in the same way as was proposed for HH16 in Figure 5. Just as discussed above for HH16 mutations, the slower k_{obs} values for the nHH8 mutations can be understood by the fact that they stabilize the inactive minimal conformation by forming stable pairing interactions between N3 and N17 or between N8 and N13 that compete with the C3–G8 pair required for activity. For example, the G3C8 mutation in nHH8, which could make a stable G3–C17 pair in the minimal hammerhead structure, has a $k_{\text{obs}} = 0.017 \text{ min}^{-1}$ and a $f_{\text{eq}} = 0.16$. If we assume that cleaved and uncleaved nHH8 are also in fully active conformations in the 1 mM MgCl_2 (pH 6.0) buffer, then its $k_{\text{obs}} = 2.5 \text{ min}^{-1}$ and $f_{\text{eq}} = 0.05$ means that $k_2 = 2.375$ and $k_{-2} = 0.125$. One can then calculate $K_u = 0.006$ and $K_c = 45$ for the G3C8 mutation. This would imply that only 0.6% of the uncleaved and 2% of the cleaved G3C8 hammerheads are in an active conformation. Since these estimates rely on the unproven assumption that nHH8 is fully active, they are mostly useful for illustrating that the changes in k_{obs} and f_{eq} reflect independent properties of each mutation. A reduced k_{obs} indicates an increased population of inactive conformations compared with wild type. However, since the expression for f_{eq} contains both K_u and K_c , f_{eq} can either increase or decrease depending upon the amount that the mutation alters each isomerization relative to wild type. It is interesting that for most of the nHH8 mutations, f_{eq} is greater than the wild-type value of 0.05, meaning that the proportion of uncleaved active molecules is reduced by more than the cleaved ones. In those cases where f_{eq} is less than 0.05, the opposite may be true, but one cannot rule out the possibility that the mutation results in trapping a fraction of the molecules in a state that never can reach an active conformation during the time course of the experiments. Further experiments measuring the corresponding cleavage reaction would help to resolve this point for these mutants.

If the k_{obs} and f_{eq} values of mutants of nHH8 can be explained by the dynamic equilibrium between multiple inactive forms and a single active form, it is possible that nHH8 itself may not always be in the active configuration.

This would mean that the k_{obs} of nHH8 may not simply equal to $k_2 + k_{-2}$, but could still contain a contribution from one or both isomerization equilibria. For example, it is known that the k_{obs} of extended hammerheads increase with magnesium ion concentration, often without evidence of saturation (Canny et al. 2004; Nelson et al. 2005). While the reason for this is not well understood, it is possible that the increase could partially be due to one or more magnesium ions further stabilizing the active conformations. Since hammerhead activity will require positioning of catalytic functional groups and possible catalytic cations prior to cleavage, any interaction adding stability to pretransition state structures will increase the rate of catalysis. It is possible that the loop–bulge or loop–loop tertiary elements could have no direct role in the catalytic mechanism but simply increase the population of molecules with the requisite energy to reach the transition state (Rueda et al. 2003). This would explain why the tertiary elements are not phylogenetically conserved (Flores et al. 2001) and can be replaced by other stabilizing tertiary elements uncovered by in vitro selection experiments (Saksmerprome et al. 2004). Indeed, the tertiary structural elements of different natural hammerheads may have evolved to have values of K_u and K_c that adjust k_{obs} and/or f_{eq} to the value it needs for its physiological function. Thus, rapid conformational switching between active and inactive states could be a critical property in the evolution of hammerheads.

MATERIALS AND METHODS

RNA oligonucleotides

HH16 and the three double-mutant ribozyme strands were transcribed using T7 RNA polymerase (Milligan and Uhlenbeck 1989) from double-stranded DNA made by extending two overlapping synthetic oligonucleotides with Taq DNA polymerase. nHH8 and the three double-mutant hammerheads were transcribed in a similar fashion and allowed to cleave in the in vitro transcription reaction (Nelson et al. 2005). Transcription products were gel purified on 10% (w/v) polyacrylamide gels and eluted overnight at 4°C in 0.5 M NaOAc and 1mM EDTA (pH 5.5), ethanol precipitated, and resuspended in water. The G3 and C8 single point mutants of nHH8 and HH16 and the substrate strands for the HH16 reaction (5′-GGGAACGUNGUCGUCGC, underlined nucleotide N17=C, A, U, or G) were purchased from Dharmacon, deblocked, and purified as described above. HH16 substrate strands were 5′ end-labeled using $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ (~6000 Ci/mmol) and T4 polynucleotide kinase. The reactions were then passed through G-25 spin columns (Amersham Biosciences) to remove excess $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ and exchange the buffer into water. The cyclic phosphate substrates (GGGAACGUN>p; N>p=C>p, A>p, U>p, or G>p) required for nHH8 ligation reactions were prepared by mixing the corresponding labeled HH16 substrate with HH16 ribozyme (pH 7.5) and 10 mM MgCl_2 and were thermocycled as described (Nelson et al. 2005). The reactions were then passed through G-25 spin columns (Amersham Biosciences) to exchange buffer with water.

Rate measurements

Cleavage reactions of wild-type and double-mutant HH16 ribozymes with 17mer substrates were carried out essentially as previously described (Hertel et al. 1994, 1996; Stage-Zimmermann and Uhlenbeck 1998). Reaction mixtures containing saturating (2 μ M) ribozyme were mixed with trace (< 5 nM) [5'-³²P]-labeled substrate in 100 mM Hepes (pH 7.5), heated to 95°C for 2 min, and then allowed to slow cool to 40°C over 1 h to allow annealing. Reactions were then spun down and equilibrated for 10 min at 25°C. Two-microliter aliquots were initiated with 20 μ L of 100 mM Hepes (pH 7.5) and 20 mM MgCl₂. Time points (3 μ L) were quenched in 15 μ L of stop solution (50 mM EDTA, 7 M Urea, $< 0.02\%$ dyes) and held on ice (or frozen). Reactions were fractionated on 20% (w/v) denaturing PAGE, and products were detected with a Molecular Dynamics Storm Phosphorimager (Amersham Biosciences) and quantified using ImageQuant (Amersham Biosciences). The data were fit by a single exponential to a k_{obs} and a f_{eq} , the fraction of full-length molecules at equilibrium, as described previously (Stage-Zimmermann and Uhlenbeck 1998). For the five mutants that did not reach completion during the time course of the experiment, it was assumed that $f_{\text{eq}} = 0.30$, which is the average of f_{eq} values obtained for the other mutants. An alternate approach to estimate f_{eq} for these five very slow mutants was to determine the fraction of full-length remaining after 14 d of incubation. This value was consistently within ± 0.1 of 0.3. Since a ± 0.1 error in f_{eq} would lead to a $\pm 20\%$ error in the corresponding k_{obs} , the errors on k_{obs} for these six mutants may be somewhat greater than the standard deviations suggest.

For thermocycling experiments on HH16(G17), cleavage reactions were initiated in 10 mM MgCl₂, 100 mM Hepes (pH 7.5) at 25°C as described above. After 3 min at 25°C, the temperature was increased for 1 min to 65°C. After taking an aliquot, the reaction was returned to 25°C to start the next cycle. A control reaction omitted the 65°C incubation. Aliquots were quenched in 5 $\times$ stop solution and analyzed as above.

Cleavage kinetics for the G3 and C8 single point mutants of HH16 were performed in 100 mM Tris-HCl (pH 8.1) and 100 mM MgCl₂. In order to compare these rates to the values of k_{obs} of the other mutants, a correction to account for the pH and MgCl₂ ion differences was needed. Since the log of k_{obs} is known to vary linearly with both pH (Hertel and Uhlenbeck 1995) and the log of the magnesium ion concentration (O'Rear et al. 2001), the k_{obs} values for the single mutants were divided by a factor of 4 to account for the difference in pH and a factor of 10 to account for the difference in the magnesium ion concentration.

Ligation reactions of wild-type nHH8 and its mutants with the 9mer substrates containing the terminal 2',3' C, A, U, or G cyclic phosphate were carried out as described above except for variations in buffer conditions and annealing procedure: 2 μ M ribozyme was mixed with < 5 nM [5'-³²P]-labeled substrate in 50 mM Mes (pH 6.0), heated to 95°C for 2 min, placed on ice for 2–6 min, spun down, and equilibrated at 25°C for 10 min; 20 μ L aliquots were then placed in a 96-well plate and reactions were initiated with 20 μ L of 50 mM Mes (pH 6.0) and 2 mM MgCl₂; and 3 μ L time points were quenched and analyzed in the same way as the HH16 reactions to give values of f_{eq} and k_{obs} . The G3 and G8 single point mutants were performed in 50 mM Hepes (pH 7.5) and 10 mM MgCl₂. In order to compare these values to the k_{obs} of

the double mutants, a correction to account for the pH and MgCl₂ differences was needed. Again, since the log of k_{obs} for nHH8 is known to vary linearly with pH and with the log of magnesium ion concentration with a slope of two (I. Shepotinovskaya, unpubl.), k_{obs} was divided by 32 to account for the difference in pH and by 100 to account for the difference in magnesium ion concentration.

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