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Morphing the minimal and full-length hammerhead ribozymes: implications for the cleavage mechanism

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Abstract

The hammerhead ribozyme is a small, intensively studied catalytic RNA, and has been used as a prototype for understanding how RNA catalysis works. In 2003, the importance of a set of tertiary contacts that appear in natural sequences of the hammerhead RNA was finally understood. The presence of these contact regions in stems I and II in 'full-length hammerhead ribozymes' is accompanied by an up to 1000-fold catalytic rate enhancement, indicating a profound structural effect upon the active site. Although the new structure resolved most of what appeared to be irreconcilable differences with mechanistic studies in solution, it did so in a way that is simultaneously reconcilable with earlier crystallographic mechanistic studies, within the limits imposed by the truncated sequence of the minimal hammerhead. Here we present an analysis of the correspondence between the full-length and minimal hammerhead crystal structures, using adiabatic morphing calculations that for the first time test the hypothesis that the minimal hammerhead structure occasionally visits the active conformation, both in solution and in the crystalline state in a sterically allowed manner, and argue that this is the simplest hypothesis that consistently explains all of the experimental observations.

Keywords: adiabatic morphing; catalytic RNA; conformational change; hammerhead ribozyme; mechanism.

Introduction

The hammerhead ribozyme is a small, self-cleaving RNA that is often regarded as a prototype for understanding ribozyme catalysis. In 1994 and 1995, two independent crystal structures (Pley et al., 1994; Scott et al., 1995b) of a minimal hammerhead ribozyme sequence appeared that were in close agreement, but unable to account for a growing body of predicted interactions (McKay, 1996; Wedekind and McKay, 1998; Blount and Uhlenbeck, 2005) based on several biochemical approaches. Nevertheless, it had been demonstrated that the minimal hammerhead ribozyme sequence crystallized was able to undergo a self-cleavage reaction in the crystal (Scott et al., 1996), and to do so to a greater extent and slightly faster than the corresponding reaction under similar conditions in solution (Murray et al., 2002). The two sets of observations appeared to be hopelessly irreconcilable. Then a more recent 2.2-Å-resolution crystal structure of a full-length hammerhead RNA (Martick and Scott, 2006) emerged. Until 2003 (De la Pena et al., 2003; Khvorova et al., 2003), it was not fully appreciated that tertiary contact regions distant from the active site could greatly enhance catalysis, and these tertiary contacts had been eliminated from the minimal hammerhead ribozyme constructs (Uhlenbeck, 1987; Haseloff and Gerlach, 1989; Ruffner et al., 1989), which still supported the self-cleavage reaction, albeit at a 1000-fold slower rate. The structure of the full-length ribozyme includes these contacts (Martick and Scott, 2006), and this in turn stabilizes the active site in a conformation consistent with the catalytic mechanism, revealing how invariant nucleotides are positioned in the active site consistent with their previously identified roles (Han and Burke, 2005) in acid-base catalysis, explaining many, if not all, of the important discrepancies between the earlier crystal structures and biochemical experiments (Nelson and Uhlenbeck, 2006). Here we attempt to assess what went wrong, and what was done right, in a process that converged upon a consistent explanation 20 years subsequent to the discovery (Prody et al., 1986) of ribozyme catalysis in the hammerhead RNA.

The hammerhead ribozyme was derived from a small, self-cleaving genomic RNA discovered in satellites of various plant RNA virus genomes (Prody et al., 1986; Forster and Symons, 1987; Uhlenbeck, 1987; Haseloff and Gerlach, 1989) and other species (Forster et al., 1988; Ferbeyre et al., 1998; Bourdeau et al., 1999). The minimal hammerhead ribozyme consists of a conserved core of approximately 15 mostly invariant residues (Ruffner et al., 1990), and for the period between 1987 and 2003, the minimal sequence (Figure 1A) was almost exclusively the one studied using biochemical and biophysical approaches.

The hammerhead ribozyme catalyses an RNA self-cleavage phosphodiester isomerization reaction that involves nucleophilic attack of the C17 2′-O upon the adjacent scissile phosphate, producing two RNA product strands. The 5′-product, as a result of this cleavage reaction mechanism, possesses a 2′,3′-cyclic phosphate terminus, and the 3′-product possesses a 5′-OH terminus. The reaction is, therefore, in principle, reversible, as the scissile phosphate remains a phosphodiester, and may thus act as a substrate for hammerhead RNA-mediated ligation without a requirement for ATP or a similar exogenous energy source. The detailed three-dimensional mechanism by which hammerhead RNA catalysis occurs has been the subject of much debate (Wedekind and McKay, 1998; Blount and Uhlenbeck, 2005), because an increasing number of biochemical experiments designed to probe transition-state interactions invoked contacts

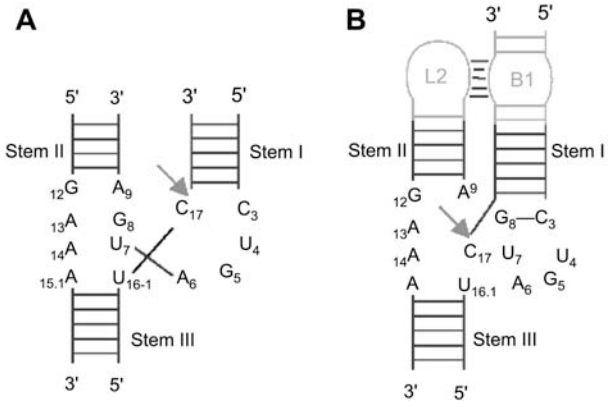

Figure 1 Minimal (A) and full-length (B) hammerhead ribozyme sequences.
The cleavage site is indicated with an arrow, and core nucleotides are shown explicitly. Stems I, II and III are represented as ladder rungs. The tertiary contact regions of the full-length hammerhead ribozyme (B) are indicated as a gray loop (L2) and a bulge (B1) that form tertiary interactions.

that appeared to be impossible based on the crystal structures of the minimal hammerhead ribozyme.

For example, the invariant residues G5, G8, G12 and C3 in the minimal hammerhead ribozyme are so fragile that changing even a single exocyclic functional group on any one of these nucleotides results in a dramatic reduction in or abolition of catalytic activity (McKay, 1996), yet few of these appeared to form hydrogen bonds involving the Watson-Crick faces of these nucleotide bases. G8 and G12 were subsequently identified (Han and Burke, 2005) as possible participants in acid/base catalysis (once it was demonstrated that the RNA itself, rather than divalent metal ions, must play this role; Murray et al., 1998a; Scott, 1999), yet it was unclear how they might accomplish this, given the minimal hammerhead ribozyme structure. A nuclear Overhauser effect (NOE) between U4 and U7 of the cleaved hammerhead ribozyme (Simorre et al., 1997) suggested that these nucleotide bases must approach one another more closely than ~6 Å, although this did not appear to be possible from the crystal structure. The attacking nucleophile, the 2′-OH of C17, was not in a position amenable to in-line attack upon the adjacent scissile phosphate (Pley et al., 1994), although it has been well established that the reaction proceeds via configuration inversion (van Tol et al., 1990; Slim and Gait, 1991). Perhaps most worrisome was the suggestion that the A-9 and scissile phosphates must come within ~4 Å of one anther in the transition state; based on double phosphorothioate substitution and soft metal ion rescue experiments (Wang et al., 1999); the distance between these phosphates in the crystal structure was ~18 Å, with no clear mechanism for close approach if the stem II and stem I A-form helices were treated as rigid bodies. Taken together, these results appeared to suggest that a fairly large-scale conformational change must take place to reach the transition state of the minimal hammerhead ribozyme structure.

Although it was apparent from the beginning that, at a very minimum, a structural rearrangement dramatic enough to bring the attacking nucleophile, the 2′-OH of C17, in line with the scissile bond must be required (Pley et al., 1994), it was not immediately apparent from the first crystal structures how this might happen. To address this requirement, conditions were obtained in which an active, unmodified minimal hammerhead ribozyme could be crystallized prior to cleavage (Scott et al., 1996). This allowed the cleavage reaction to be monitored and assayed in the crystal (Murray et al., 2002), and permitted crystallographic freeze-trapping experiments that arrested conformational changes occurring prior to the cleavage reaction (in some cases assisted by modifications of the RNA) (Scott et al., 1996; Murray et al., 1998b; Dunham et al., 2003). The hammerhead RNA sequence crystallized had not been optimized for catalysis (Scott et al., 1995a), but cleaved approximately five-fold faster in the crystal (~0.4 /min at pH 8) than in solution, and to a greater extent (95% in the crystal vs. ca. 75% in solution) (Murray et al., 2002). These observations, along with trapped structures that brought the attacking nucleophile within ~35° of an in-line orientation (Dunham et al., 2003; Murray et al., 1998b), appeared to suggest torsion angle conformational changes involving the β-backbone angles of C17 and the nucleotide at position 1.2 (two units 3′ to the cleavage site) might alone be sufficient to position C17 for in-line attack. Hence, although it was well understood that a conformational change involving C17 would be required to reach the transition state (Pley et al., 1994; Scott et al., 1995b), it appeared that the minimal hammerhead RNA crystal structure, including the lattice contacts involving the distal ends of stems I, II and III, was at least consistent with the global fold of the active hammerhead RNA (Murray et al., 2000). For these reasons, the two sets of experiments (biochemical vs. crystallographic) appeared to be not only at odds, but also irreconcilable, generating a substantial amount of discord in the field (Blount and Uhlenbeck, 2005). No compelling argument for dismissing either set of experimental results was ever successfully produced, although many claims to the contrary (Wang et al., 1999; Murray and Scott, 2000; Blount and Uhlenbeck, 2005) were made in favor of each.

Then, when all seemed a hopeless morass, in 2003 it was finally pointed out that for optimal activity, the hammerhead ribozyme requires the presence of sequences in stems I and II that interact to form tertiary contacts (De la Pena et al., 2003; Khvorova et al., 2003) (Figure 1B), making it clear that a crystal structure of the full-length hammerhead ribozyme in which these distal tertiary contacts were present might be of some interest. We subsequently obtained a 2.2-Å-resolution crystal structure of the full-length hammerhead ribozyme (Martick and Scott, 2006). This new structure of the full-length hammerhead ribozyme appears to resolve the most worrisome of the previous discrepancies. In particular, C17 is now positioned for in-line attack, and the invariant residues C3, G5, G8 and G12 all appear to be involved in vital interactions relevant to catalysis. Moreover, the A9 and scissile phosphates are observed to be 4.3 Å apart, consistent with the idea that, when modified, these phosphates could bind a single thiophilic metal ion. The structure also reveals how two invariant residues, G12 and G8,

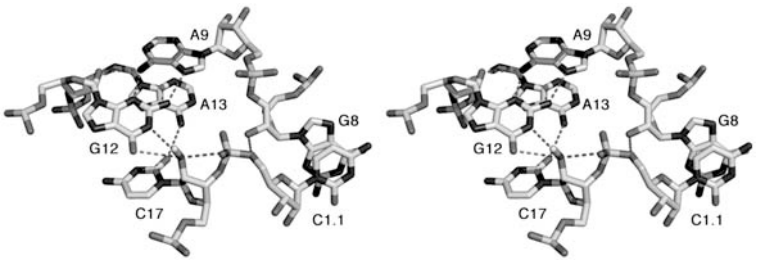

Figure 2 Active site of the hammerhead ribozyme.
Stereoview of the arrangement of active-site residues in the full-length hammerhead ribozyme structure. The nucleotides are numbered according to the canonical scheme, where C17 is the cleavage-site nucleotide, G12 is positioned for general base catalysis, and the 2′-OH of G8 appears to be involved in general acid catalysis. The 2′-OH is positioned almost perfectly for an in-line attack (prevented by the 2′-OMe modification of C17). Close contacts that appear catalytically relevant are indicated with dotted lines.

are positioned within the active site, consistent with their previously proposed (Han and Burke, 2005) role in acid/base catalysis. G12 is within hydrogen bonding distance to the 2′-O of C17, the nucleophile in the cleavage reaction, and the ribose of G8 hydrogen bonds to the 5′-O leaving group (Figure 2), while the nucleotide base of G8 forms a Watson-Crick pair with the invariant C3. This arrangement permits the suggestion that G12 is the general base in the cleavage reaction, and that G8 may function as the general acid, consistent with previous biochemical observations (Han and Burke, 2005). G5 hydrogen bonds to the furanose oxygen of C17, helping to position it for in-line attack. U4 and U7, as a consequence of the base-pair formation between G8 and C3, are now positioned such that an NOE between their bases is easily explained.

The crystal structure of the full-length hammerhead ribozyme thus clearly addresses all of the major concerns that appeared irreconcilable with the previous crystal structure (Nelson and Uhlenbeck, 2006). What is less obvious, and what has not been addressed to a significant extent, is whether and how the new crystal structure can be reconciled with the previous, minimal hammerhead structures. Here we attempt to do this, and in so doing, assess what went wrong, and what was on the right track, with the previous structural analyses, from the point of view of the full-length hammerhead structure.

Results and discussion

Does the minimal hammerhead ribozyme rely upon the same cleavage mechanism as the full-length hammerhead? If this is the case, then it must be possible for the minimal hammerhead ribozyme structure to be continuously deformable into that observed in the full-length hammerhead. The hypothesis that the minimal hammerhead can be deformed into a full-length-like structure is computationally testable using a morphing procedure. If the morphing hypothesis is falsified, then separate cleavage mechanisms must be invoked to explain minimal vs. full-length hammerhead catalysis or, in other words, the two would have to be considered different ribozymes.

Several mutually exclusive but testable hypothetical relationships that might exist among the various hammerhead crystal structures can be envisioned. One possibility is that each structure listed in Table 1 represents a unique, sequential conformational state on the cleavage reaction pathway for the minimal hammerhead ribozyme, in which State 1 is the initial, uncleaved state, State 2 represents an activated, early conformational intermediate, State 3, based on the full-length ribozyme structure, represents a near-transition-state pre-cleavage structure, and State 4, the cleaved minimal hammerhead, represents the final state in the cleavage reaction sequence. This, in essence, is the hypothesis that the various states represent relevant, sequential, on-pathway structures in the minimal hammerhead cleavage reaction mechanism, and that each state can be continuously deformed into the subsequent state with torsion-angle conformational adjustments of relatively low energy barrier (apart from the scissile bond that must break as the ribozyme cleaves).

A plausible, competing hypothesis is that not all of these states are consistent in the sense that it might not be sterically possible to deform the Nth state into the (N+1)th state. More specifically, the hypothesis that the minimal hammerhead ribozyme can at least occasionally visit the conformation represented by the full-length hammerhead active site, and therefore undergoes the cleavage reaction according to the same chemical mechanism as that for full-length hammerhead, critically depends on

Table 1 Crystal structures used as adiabatic morphing end-points.

State	PDB code	Description	Reference
1	299D	Uncleaved initial state of the minimal hammerhead ribozyme (with stem III GAAA tetraloop removed)	Scott et al., 1996
2	1Q29	Uncleaved pre-cleavage conformational intermediate minimal hammerhead	Dunham et al., 2003
3	2GOZ	Uncleaved full-length hammerhead (with peripheral sequences deleted and base changes implemented to match 299D (with stem III GAAA tetraloop removed)	Martick and Scott, 2006
4	488D	Cleaved product state of the minimal hammerhead ribozyme (with stem III GAAA tetraloop removed)	Murray et al., 2000

whether State 1 and/or State 2 can be continuously deformed to arrive at State 3. The adiabatic morphing experiments are designed to test this hypothesis.

Morphing between minimal hammerhead structures

As a set of positive controls, we first morphed the initial state directly into the final state of the minimal hammerhead ribozyme (States 1 and 4). Then we morphed State 1 into State 2 (the conformational intermediate), followed by morphing State 2 into State 4. The object of these morphing experiments was to observe what aspects of the full-length structure might be predicted by the morphing computations in the absence of any prior structural knowledge of the full-length hammerhead conformation.

Morphing directly between states 1 and 4 Using only the initial-state structure (State 1) and the cleaved structure (State 4), can we predict the cleavage reaction path? Will any aspects of the full-length hammerhead ribozyme that appear in this trajectory contradict prior knowledge of the active site structure of the full-length hammerhead?

The morphing software is in principle free to choose any pathway that connects the two known endpoints (States 1 and 4), constrained only by steric and energetic restrictions. No knowledge of the S_N2 reaction mechanism or of the intermediate conformation is incorporated in this analysis. It should be noted that this treatment has some obvious limitations, including the fact that it is unable to reproduce non-equilibrium phosphate geometries (i.e., intermediate or transition-state structures that have significant trigonal bipyramidal phosphate character). Nonetheless, we believed it would be informative to identify what global conformational changes might occur that accompany the transition from reactants to products using only information obtained from the minimal hammerhead structures.

We find that when using only the initial- and final-state minimal hammerhead structures in the morphing analysis, the structure of the hammerhead just prior to cleavage has some striking similarities to what is observed in the full-length hammerhead ribozyme, which is thought to be a structure rather close to that adopted in the transition state (Figure 3). The main differences are that the G8-C3 base pair does not form, with the consequence that the G12 and G8 residues are not poised for acid-base catalysis, and the relative disposition of stem I with respect to the rest of the ribozyme does not change as dramatically as observed in the full-length hammerhead structure. The orientation of the cleavage-site base, however, is strikingly similar to what is observed in the full-length hammerhead structure, and the 2′-OH nucleophile is oriented in a conformation amenable to in-line attack in much the same way as is observed in the full-length hammerhead.

The morphing protocol employed in this analysis only allows interpolation between two conformational states of the same covalent entity. The software has not been told to break or make any bonds, and it also assumes that the phosphodiester linkage has to be constrained throughout the course of the conformational change in an equilibrium (tetrahedral) geometry. The chemical reac-

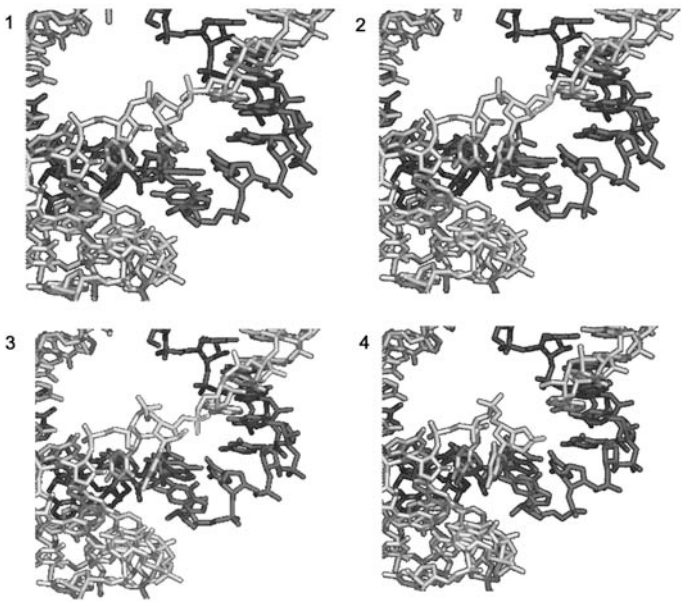

Figure 3 Morphing the minimal hammerhead ribozyme.
The morphing trajectory of the minimal hammerhead ribozyme is illustrated as a four-frame progression, with the enzyme strand shown as dark gray and the substrate strand light gray. The first frame (1) depicts the cleavage site base, C17, in a conformation that recapitulates much of the (omitted) State 2 intermediate crystal structure. The main difference is that the scissile phosphate is oriented in the direction opposite from that observed in the State 2 crystal structure. The next frame (2), slightly further along the reaction pathway, reveals that the phosphate becomes rather distorted as it is about to invert. The orientation of C17 is rather similar to that observed in the full-length hammerhead structure. Frame 3 depicts the morphing state that immediately follows that shown in frame (2). The phosphate has inverted and the conformation now appears consistent with what is observed in the crystal structures. The last frame (4) depicts the cleavage product, with a 2′,3′-cyclic phosphate.

tion, by contrast, requires an inversion of configuration in the phosphate, accompanied by breakage of the scissile bond, and to do this the actual reaction must pass through a pentacoordinated intermediate or transition state that is by definition incompatible with the above simplistic assumptions.

Given these restrictions, it is actually quite surprising that the morphed trajectory even remotely resembles what we have proposed based on the various crystal structures, including the cleavage product. We therefore examined the scissile phosphate in the end-point morphing interpolation by considering several frames from the resulting movie.

It is important to note that the existence of the 2′-P bond was not accounted for in the morphing program. The fourth frame in Figure 3 was rendered manually to remove a highly elongated P to O′3 bond and the 2′-P bond was manually added. Hence the conformational state of the endpoint structure, and not the cyclic phosphate, forced the program to perform the phosphate inversion. Remarkably, with no prior knowledge of chemical mechanism of cleavage, considering only a trajectory calculated by morphing the initial-state conformation into the final-state conformation forces prediction of inversion of the scissile phosphate. There is exactly one energetically plausible mechanism by which a phosphate can invert: a phosphodiester bond isomerization and backbone cleavage that passes through a trigonal bipyramidal transition state or intermediate. Therefore, the morphing trajectory that we calculated as being the most energetically accessible must be one in which the phosphodiester bond is isomerized. The morphing software, despite its limitations, predicts that this trajectory is one that entails a cleavage reaction.

Morphing between States 1 and 4 with State 2 as an intermediate If we now consider a trajectory defined by three conformational states of the minimal hammerhead ribozyme, specifically, States 1, 2 and 4 in Table 1, we can produce a single 64-frame movie depicting a smooth transition between the initial-state, pre-catalytic intermediate and product states. Analyzing the individual coordinate sets corresponding to frames 19 and 31 by superimposing those predicted structures onto actual crystallographic intermediate structures (PDB structures 301D and 379D) (Scott et al., 1996; Murray et al., 1998b) reveals that two crystallographically observed intermediate conformational structures of the minimal hammerhead ribozyme (that were not included in the morphing calculations) are predicted to a high degree of accuracy, as shown in Figure 4. This represents an important positive control, as the ability to predict intermediate State 2 with a high degree of precision indicates that these structures clearly do not deviate from a continuous, energetically favorable pathway defined by States 1, 2 and 4.

Morphing between minimal and full-length hammerhead structures

Although the structure of the full-length hammerhead ribozyme, at first glance, appears to be radically different from the minimal hammerhead, both share some similarities in structure in both the global fold and in detail. The

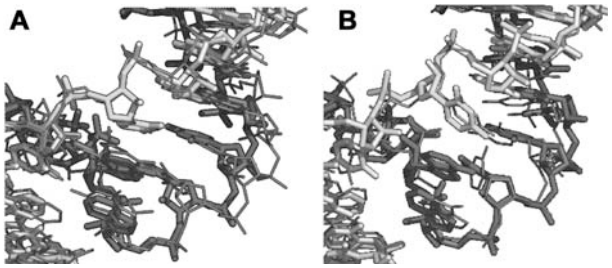

Figure 4 A morphing positive control.
(A) Frame 19 from the interpolation (enzyme strand is dark gray, substrate strand is light gray) superimposed with the 'early' conformational intermediate structure (PDB 301D; Scott et al., 1996) (shown as narrower gray wires), illustrating that the interpolated trajectory successfully predicts an observed conformational intermediate on the cleavage reaction pathway. (B) Frame 31 from the interpolation is superimposed with a 'later' conformational intermediate structure (PDB 379D; Murray et al., 1998b) (shown as narrower gray wires), illustrating that the interpolated trajectory also successfully predicts the next observed conformational intermediate on the cleavage reaction pathway.

similarities are best observed by comparing the set of nucleotides shared in common. Specifically, the similarities are most apparent by comparison of the core residues and the first five base-pairs of stem I, as well as the shared residues of stems II and III, while omitting the capping loops. A side-by-side comparison of the folds of the minimal and full-length hammerheads is shown in Figure 5. What is apparent from the comparison made in this manner, in which only the shared nucleotides are considered, is that the folds are strikingly similar, the largest difference being the kink in the substrate strand at the cleavage site that accompanies rearrangement of the active-site nucleotides.

The significance of this observation is that it explains why the hammerhead ribozyme cleavage reaction could take place in crystals of the minimal construct (Scott et al., 1996). These crystals, being 78% solvent by vol-

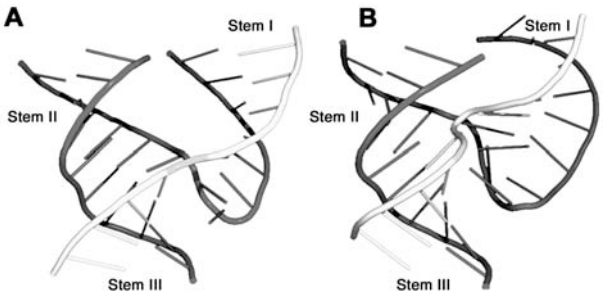

Figure 5 A familiar fold.
Comparison of the minimal (A) and full-length (B) hammerhead ribozyme folds. Only the nucleotides in common are shown. The substrate residues are shown in light gray, apart from the cleavage-site nucleotide, which is slightly more shaded. The enzyme strand is darker gray. Stems I, II and III are labeled. The crystal contacts in the minimal hammerhead structure have the effect of constraining the positions of the distal ends of each of these stems. Stem I of the full-length structure is unwound by approximately 1/4 turn relative to the minimal structure, and the substrate is severely kinked in the vicinity of the cleavage site. Apart from these differences, the folds are remarkably similar.

ume, permit molecular motions to take place subject to constraints imposed by the crystal lattice contacts. The lattice contacts of the minimal hammerhead restrict the distal termini of stems I, II and III (Scott et al., 1996).

In solution, the simplest explanation for all of the observed minimal hammerhead biochemistry (including the invariance of G5, G8, G12, C3 and the proximity of the A9 and scissile phosphates, as well as the 1000-fold slower cleavage rate of the minimal hammerhead) is that the active conformational state, which resembles the structure of the full-length hammerhead, occurs only transiently, such that only ~0.1% of the uncleaved molecules occupy this state at any given time. Thus, for cleavage to occur, a transient conformational change must take place that deforms the structure observed in the minimal hammerhead crystals into that resembling the full-length hammerhead, in which the nucleotides critical for catalysis are correctly positioned.

This rearrangement can in fact take place, because only the distal ends of the three helical stems are restricted in movement. Alternative hypotheses, including the suggestion that the minimal hammerhead cleaves via a different pathway, and that the minimal hammerhead structure in solution is identical to the full-length hammerhead conformation observed in the crystal structure, have less explanatory power. The first hypothesis cannot explain the requirement for the invariant residues, and the second hypothesis cannot account for the observed 1000-fold rate enhancement. Hence, it seems most likely that in solution, the minimal hammerhead has the same structure as observed in the crystal, and that in both cases it visits the conformation stabilized in the full-length hammerhead construct.

To test the hypothesis that the minimal hammerhead ribozyme could undergo a conformational change to the active state, we attempted to deform the minimal hammerhead crystal structure into the conformation consistent with the full-length hammerhead crystal structure. Again using adiabatic morphing (Krebs and Gerstein, 2000) implemented within the macromolecular refinement algorithm of CNS (Brunger et al., 1998a), we were able to demonstrate that the structure observed in the minimal hammerhead ribozyme can be continuously deformed via low energy-barrier torsion angle conformational changes of into the structure observed in the full-length hammerhead. This process is best represented as a series of consecutive structures viewed as a movie (see Figure 6 and the accompanying link).

The morphing analysis not only reveals that a transient conformational change within the context of the minimal hammerhead structure can allow cleavage to take place, but predicts that crystal packing will have a somewhat inhibitory effect upon cleavage, since relative to stems II and III, stem I must twist by approximately one-quarter of a helical turn, as can be clearly observed in the context of the movie. When assaying cleavage activity in the crystal, it became apparent that the turnover rate was biphasic, with a transition from a slower to faster phase occurring at the point at which 50% of the substrate was cleaved (Murray et al., 2002). (The fast phase at pH 6 corresponds to the rate observed for hammerhead α1, the fastest minimal hammerhead construct.) A plausible interpretation is that upon cleavage, the constraints imposed by the lattice contacts become more flexible due to strand breakage, and at the 50%-cleaved point, the crystal lattice collectively ceases to be inhibitory.

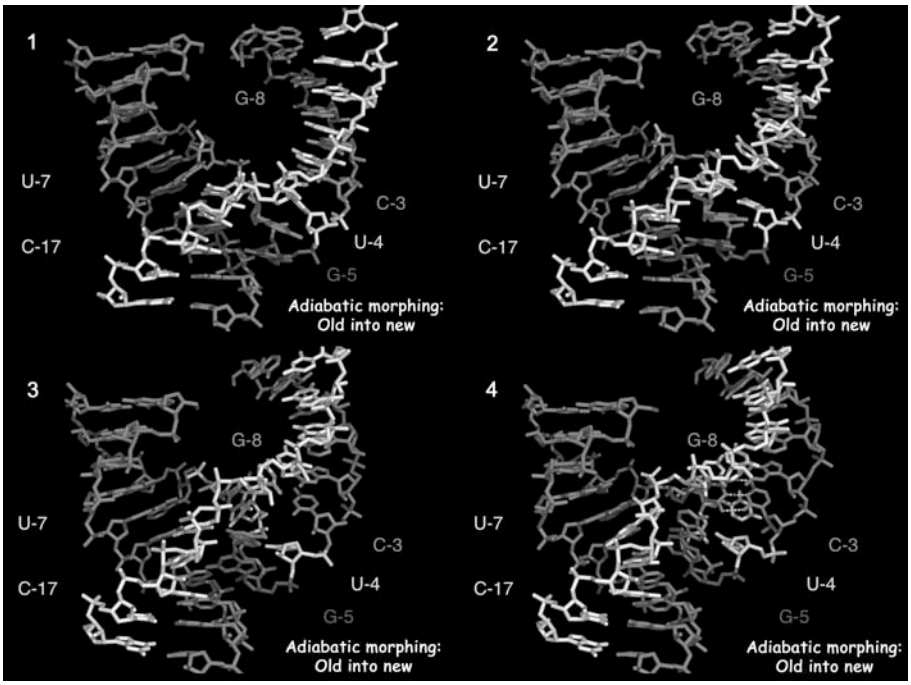

Figure 6 Morphing movie.
Four 'frames' from a movie produced by adiabatically morphing the common nucleotides from the minimal hammerhead ribozyme structure (step 1) into the full-length hammerhead ribozyme (step 4). The actual movie is available in QuickTime at the following site: http://xanana.ucsc.edu/hh/figs/biological_chemistry/Figure6_biological_chemistry.mov.

Assessing the observations of cleavage in the crystal

Assuming the above analysis is accurate, we can understand not only why it was possible to observe cleavage in the crystal, but also can assess both the successes and shortcomings of this approach.

For the reasons previously stated, it is likely that the first, initial-state hammerhead ribozyme crystal structures represent more or less accurately the dominant structure of the minimal hammerhead ribozyme in solution. As mentioned, this suggestion explains why the minimal hammerhead is 1000-fold less active in solution than the full-length hammerhead. Since the crystal lattice appears to have both inhibitory and enhancing effects, it is likely that the minimal hammerhead in solution is quite dynamic and flexible.

The cleaved state of the minimal hammerhead in some ways resembles the full-length hammerhead to a greater extent than does the uncleaved minimal hammerhead structure. Specifically, in the cleaved structure (Murray et al., 2000), it was observed that the cleavage-site base, C17, makes contacts with G5 and A6 that are similar to those observed in the full-length structure, and the interactions with C3 are completely absent in both cases. The cleavage intermediates, in retrospect, appear to resemble a torsion-angle conformational change of only approximately one-third of what is required to morph the structure from the minimal hammerhead to the full-length hammerhead active-site conformation. If the intermediate is representative of an on-pathway state, this would suggest that alignment of the attacking nucleophile (possibly accompanied by deprotonation) with the scissile phosphate occurs comparatively early in the transition. If so, this would be consistent with our observation that the conformational change, rather than the chemical step, is the rate-limiting, pH-dependent step in minimal hammerhead ribozyme catalysis (Murray et al., 2002).

In summary, the crystallographic observations of various states along the cleavage reaction pathway appear to be more incomplete than erroneous. Missing from the set was the low-occupancy transient conformation that is stabilized by the distal tertiary contacts in the full-length hammerhead ribozyme. In crystallographic experiments, one can only hope to resolve the dominant species in the population, so it is likely that the true pre-catalytic intermediate would never be observed crystallographically in the context of the minimal hammerhead construct.

Assessing predictions based upon the crystal structures

In 2000 we published a paper entitled ‘Does a single metal ion bridge the A9 and scissile phosphates?’ (Murray and Scott, 2000). The main conclusion, based on modeling studies using the hammerhead crystal structures and rigid A-form RNA helical stems, was that this could not happen. We took as assumptions the idea that metal ion binding required non-bridging phosphate oxygens to approach within ~4 Å of one another such that the two metal-oxygen bonds required would form an angle that would be either 90° or 180°, and that the A-form RNA helices that comprised stems I, II and III, and the structure that comprised the augmented stem II helix (or Domain II) could be treated as rigid bodies, apart from the phosphate linkages at their ends. These assumptions were carefully and explicitly stated toward the end of the paper as the ‘minimum set of assumptions that leads to a contradiction of the hypothesis.’ They seemed to be quite reasonable at the time. In particular, ‘any model structure compatible with the crosslinking data’ (i.e., the requirement that the two phosphates in question approach closely enough to bind a single metal ion) ‘must involve significant disruption of the helices’ either through unpairing (fraying) or unwinding. Implicit in this statement was the assumption that such an event was rather unlikely.

The analysis, given these seemingly reasonable assumptions, was in fact internally consistent. Unfortunately, it was irrelevant, since one of the two sets of assumptions was unwarranted and clearly wrong. (The other, based purely on Mg^{2+} coordination chemistry, was in retrospect a safe assumption.) In particular, G8 rather dramatically unpairs with A13 to instead form a Watson-Crick pair with C3. This simultaneously ‘frays’ or unpairs the augmented stem II helix by one pairing unit and lengthens stem I by one base pair. (In addition, stem I becomes rather underwound relative to the minimal structure.) In other words, the assumption that stems I and II would retain their original base-pairing schemes as the minimal hammerhead ribozyme approached the transition state proved to be the fatal flaw of this analysis. It was based on the assumption that base-pair fraying is energetically costly, but the base-pair switch observed is probably close to isoenergetic, since one pair (a G/A pairing interaction) is lost from stem II and one pair (a Watson-Crick G/C pair) is gained at the base of stem I.

Concluding remarks

In summary, it appears that actual experimental data obtained from crystallographic analyses and biochemical characterizations, which were performed on high-occupancy, near-ground-state and transient near-transition-state structures, respectively, were sound within the confines imposed by the minimal hammerhead structure. The mutually held interpretation that acceptance of one set of experimental results precluded acceptance of the other, however, was based on the flawed assumption that the two sets of observations were incommensurate and irreconcilable. In our case, the flawed assumption manifested itself most explicitly as the claim that unwinding and unpairing of helical elements was unlikely to take place (Murray and Scott, 2000). In the other case, the flawed assumption manifested itself with the claim that any cleavage observed in the crystal must be due to an off-pathway artifact or experimental incompetence (Wang et al., 1999; Blount and Uhlenbeck, 2005). In retrospect, neither dismissal was justified or compelling. The resolution of the apparent paradox came with the structure of the full-length hammerhead, which reconciles and explains both sets of experimental results.

Materials and methods

Crystallographic coordinates

Coordinates in PDB format for four hammerhead structures previously determined in our laboratory were obtained from the protein data bank. These included the unmodified minimal hammerhead ribozyme initial-state structure (299D) and the minimal hammerhead ribozyme conformational intermediate structure (1Q29), the full-length hammerhead ribozyme structure (2OEU), and the minimal hammerhead ribozyme cleavage product (488D) structure. These are summarized and referenced in Table 1.

Software

The adiabatic morphing calculations were performed within the crystallographic software refinement package CNS 1.2 (Brunger et al., 1998b) using a programmed input file for adiabatic morphing calculations (morph_dist.inp) developed and kindly provided by Nat Echols and the other authors of the Yale Morph Server (Krebs and Gerstein, 2000; Echols et al., 2003; Flores et al., 2006). Editing and idealization of the starting models was performed using COOT (Emsley and Cowtan, 2004), and molecular morphing movies were rendered in PyMOL (DeLano, 2002).

General morphing procedure

The morphing procedure requires two known structures (in the form of PDB files) as endpoints and interpolates an energetically favorable trajectory between them (Krebs and Gerstein, 2000), yielding an approximately smooth transition consisting of a preselected number (e.g., 30) of discrete steps. Each of these discrete steps is recorded as a complete PDB coordinate file that contains a geometrically idealized model, and the resulting catenation of these with the starting and endpoint coordinates results in a composite PDB file that contains an ensemble of (e.g., 32) structures, similar to what is conventionally used by NMR spectroscopists to report a family of structures consistent with experimental data. In the present case, the structures are ordered along the morphing trajectory, so that when displayed by molecular rendering software, such as PyMOL, the succession of structures appears as a movie. Molecular morphing movies can thus be displayed directly from PDB files using software such as PyMOL, or can be captured and displayed as animated gif files or QuickTime movies. We have preserved our results and have made them available publicly in all three formats.

The morphing procedure as implemented in CNS requires that both endpoint structures consist of all of the same atoms. Since the full-length hammerhead obviously possesses sequences absent in the minimal hammerhead ribozyme, and since non-conserved stem I, II and III nucleotides in the minimal hammerhead sequence differ from the corresponding nucleotides in the full-length model, the full-length model was edited in COOT to produce a model sharing all atoms in common with the minimal hammerhead (299D). This involved deleting peripheral stem I and stem II sequences, including the bulge and loop, and mutating several of the non-conserved base-pairs, followed by geometric idealization within COOT to ensure a valid starting model. The GAAA tetraloop on stem III of the minimal hammerhead structures was also deleted, as no corresponding structure exist in the full-length hammerhead. An MTF structure connectivity file required for the morphing was generated in CNS for the minimal hammerhead with the GAAA tetraloop deleted.

Specific morphing experiments

The four PDB files were edited within COOT to produce four conformational states identical in sequence and atomic composition, and differing only in conformation. These are summarized in Table 1.

Several morphing experiments were then performed. The first of these, a positive control, was simply to morph State 1 into State 4, i.e., the initial uncleaved minimal hammerhead (299D) into the cleaved minimal hammerhead structure (488D) in 30 steps. The second involved morphing State 1 into State 2 and then State 4, i.e., the initial uncleaved minimal hammerhead (299D) into the conformational intermediate minimal (1Q29) and cleavage product minimal (488D) structures, in two successive 30-step sequences. Finally, State 1, the initial uncleaved minimal hammerhead (299D), and State 2, the conformational intermediate minimal (1Q29), were each separately morphed into the corresponding subset of the full-length hammerhead structure (2GOZ), State 4, using both 30- and 60-step morphing procedures.

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References

Blount, K.F. and Uhlenbeck, O.C. (2005). The structure-function dilemma of the hammerhead ribozyme. Annu. Rev. Biophys. Biomol. Struct. *34*, 415–440.

Bourdeau, V., Ferbeyre, G., Pageau, M., Paquin, B., and Cedergren, R. (1999). The distribution of RNA motifs in natural sequences. Nucleic Acids Res. *27*, 4457–4467.

Brunger, A.T., Adams, P.D., Clore, G.M., DeLano, W.L., Gros, P., Grosse-Kunstleve, R.W., Jiang, J.S., Kuszewski, J., Nilges, M., Pannu, N.S., et al. (1998). Crystallography & NMR system: A new software suite for macromolecular structure determination. Acta Crystallogr. D Biol. Crystallogr. *54*, 905–921.

De la Pena, M., Gago, S., and Flores, R. (2003). Peripheral regions of natural hammerhead ribozymes greatly increase their self-cleavage activity. EMBO J. *22*, 5561–5570.

DeLano, W.L. (2003). The PyMOL Molecular Graphics System. DeLano Scientific, Palo Alto, CA, USA (www.pymol.org).

Dunham, C.M., Murray, J.B., and Scott, W.G. (2003). A helical twist-induced conformational switch activates cleavage in the hammerhead ribozyme. J. Mol. Biol. *332*, 327–336.

Echols, N., Milburn, D., and Gerstein, M. (2003). MolMovDB: analysis and visualization of conformational change and structural flexibility. Nucleic Acids Res. *31*, 478–482.

Emsley, P. and Cowtan, K. (2004). Coot: model-building tools for molecular graphics. Acta Crystallogr. D Biol. Crystallogr. *60*, 2126–2132.

Ferbeyre, G., Smith, J.M., and Cedergren, R. (1998). Schistosome satellite DNA encodes active hammerhead ribozymes. Mol. Cell. Biol. *18*, 3880–3888.

Flores, S., Echols, N., Milburn, D., Hespenheide, B., Keating, K., Lu, J., Wells, S., Yu, E.Z., Thorpe, M., and Gerstein, M. (2006). The Database of Macromolecular Motions: new features added at the decade mark. Nucleic Acids Res. *34*, D296–D301.

Forster, A.C. and Symons, R.H. (1987). Self-cleavage of virusoid RNA is performed by the proposed 55-nucleotide active site. Cell *50*, 9–16.

Forster, A.C., Davies, C., Sheldon, C.C., Jeffries, A.C., and Symons, R.H. (1988). Self-cleaving viroid and newt RNAs may only be active as dimers. Nature *334*, 265–267.

Han, J. and Burke, J.M. (2005). Model for general acid-base catalysis by the hammerhead ribozyme: pH-activity relationships of G8 and G12 variants at the putative active site. Biochemistry *44*, 7864–7870.

Haseloff, J. and Gerlach, W.L. (1989). Sequences required for self-catalysed cleavage of the satellite RNA of tobacco ringspot virus. Gene *82*, 43–52.

Khvorova, A., Lescoute, A., Westhof, E., and Jayasena, S.D. (2003). Sequence elements outside the hammerhead ribozyme catalytic core enable intracellular activity. Nat. Struct. Biol. *10*, 708–712.

Krebs, W.G. and Gerstein, M. (2000). The morph server: a standardized system for analyzing and visualizing macromolecular motions in a database framework. Nucleic Acids Res. *28*, 1665–1675.

Martick, M. and Scott, W.G. (2006). Tertiary contacts distant from the active site prime a ribozyme for catalysis. Cell *126*, 309–320.

McKay, D.B. (1996). Structure and function of the hammerhead ribozyme: an unfinished story. RNA *2*, 395–403.

Murray, J.B. and Scott, W.G. (2000). Does a single metal ion bridge the A-9 and scissile phosphate groups in the catalytically active hammerhead ribozyme structure? J. Mol. Biol. *296*, 33–41.

Murray, J.B., Dunham, C.M., and Scott, W.G. (2002). A pH-dependent conformational change, rather than the chemical step, appears to be rate-limiting in the hammerhead ribozyme cleavage reaction. J. Mol. Biol. *315*, 121–130.

Murray, J.B., Seyhan, A.A., Walter, N.G., Burke, J.M., and Scott, W.G. (1998a). The hammerhead, hairpin and VS ribozymes are catalytically proficient in monovalent cations alone. Chem. Biol. *5*, 587–595.

Murray, J.B., Terwey, D.P., Maloney, L., Karpeisky, A., Usman, N., Beigelman, L., and Scott, W.G. (1998b). The structural basis of hammerhead ribozyme self-cleavage. Cell *92*, 665–673.

Murray, J.B., Szoke, H., Szoke, A., and Scott, W.G. (2000). Capture and visualization of a catalytic RNA enzyme-product complex using crystal lattice trapping and X-ray holographic reconstruction. Mol. Cell *5*, 279–287.

Nelson, J.A. and Uhlenbeck, O.C. (2006). When to believe what you see. Mol. Cell *23*, 447–450.

Pley, H.W., Flaherty, K.M., and McKay, D.B. (1994). Three-dimensional structure of a hammerhead ribozyme. Nature *372*, 68–74.

Prody, G.A., Bakos, J.T., Buzayan, J.M., Schneider, I.R., and Breuning, G. (1986). Autolytic processing of dimeric plant virus satellite RNA. Science *231*, 1577–1580.

Ruffner, D.E., Dahm, S.C., and Uhlenbeck, O.C. (1989). Studies on the hammerhead RNA self-cleaving domain. Gene *82*, 31–41.

Ruffner, D.E., Stormo, G.D., and Uhlenbeck, O.C. (1990). Sequence requirements of the hammerhead RNA self-cleavage reaction. Biochemistry *29*, 10695–10702.

Scott, W.G. (1999). RNA structure, metal ions, and catalysis. Curr. Opin. Chem. Biol. *3*, 705–709.

Scott, W.G., Finch, J.T., Grenfell, R., Fogg, J., Smith, T., Gait, M.J., and Klug, A. (1995a). Rapid crystallization of chemically synthesized hammerhead RNAs using a double screening procedure. J. Mol. Biol. *250*, 327–332.

Scott, W.G., Finch, J.T., and Klug, A. (1995b). The crystal structure of an all-RNA hammerhead ribozyme: a proposed mechanism for RNA catalytic cleavage. Cell *81*, 991–1002.

Scott, W.G., Murray, J.B., Arnold, J.R., Stoddard, B.L., and Klug, A. (1996). Capturing the structure of a catalytic RNA intermediate: the hammerhead ribozyme. Science *274*, 2065–2069.

Simorre, J.P., Legault, P., Hangar, A.B., Michiels, P., and Pardi, A. (1997). A conformational change in the catalytic core of the hammerhead ribozyme upon cleavage of an RNA substrate. Biochemistry *36*, 518–525.

Slim, G. and Gait, M.J. (1991). Configurationally defined phosphorothioate-containing oligoribonucleotides in the study of the mechanism of cleavage of hammerhead ribozymes. Nucleic Acids Res. *19*, 1183–1188.

Uhlenbeck, O.C. (1987). A small catalytic oligoribonucleotide. Nature *328*, 596–600.

van Tol, H., Buzayan, J.M., Feldstein, P.A., Eckstein, F., and Bruening, G. (1990). Two autolytic processing reactions of a satellite RNA proceed with inversion of configuration. Nucleic Acids Res. *18*, 1971–1975.

Wang, S., Karbstein, K., Peracchi, A., Beigelman, L., and Herschlag, D. (1999). Identification of the hammerhead ribozyme metal ion binding site responsible for rescue of the deleterious effect of a cleavage site phosphorothioate. Biochemistry *38*, 14363–14378.

Wedekind, J.E. and McKay, D.B. (1998). Crystallographic structures of the hammerhead ribozyme: relationship to ribozyme folding and catalysis. Annu. Rev. Biophys. Biomol. Struct. *27*, 475–502.

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