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A pH-dependent Conformational Change, Rather than the Chemical Step, Appears to be Rate-limiting in the Hammerhead Ribozyme Cleavage Reaction

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We have investigated the chemical basis for a previously observed 7.8 Å conformational change in the hammerhead ribozyme that positions the substrate for in-line attack. We have found that the conformational change can only be observed at or above pH 8.5 (in the presence of Co^{2+}) and requires the presence of an ionizable 2′-OH at the cleavage site, and note that this observed apparent pK_a of 8.5 for the conformational change is within experimental error (±0.5) of the previously reported apparent kinetic pK_a of 8.5 for the hammerhead ribozyme in the presence of Co^{2+}. We have solved two crystal structures of hammerhead ribozymes having 2′-OCH_3 or 2′-F substitutions at the cleavage site and have found that these will not undergo a conformational change equivalent to that observed for the hammerhead ribozyme having an unmodified attacking nucleophile under otherwise identical conditions. We have also characterized the kinetics of cleavage in the crystal. In addition to verifying that the particular sequence of RNA that we crystallized cleaves faster in the crystal than in solution, we also find that the extent of cleavage in the crystal is complete, unlike in solution where this and most other hammerhead ribozyme substrates are cleaved only to about 70% completion. The initial cleavage rate in the crystal obeys the expected log-linear relation between cleavage-rate and pH with a slope of 0.7, as has been observed for other hammerhead ribozyme sequences in solution, indicating that in both the crystal and in solution the pH-dependent step is rate-limiting. However, the cleavage rate in the crystal is biphasic, with the most dramatic distinction between initial (slower) and final (faster) phases appearing at pH 6.0. The initial phase corresponds to the pH-dependent cleavage rate observed in solution, but the second, faster phase is roughly pH-independent and closely parallels the cleavage rate observed at pH 8 (0.4/minute). This result is particularly remarkable because it entails that the rapidly cleaving phase at pH 6 is comparable to the cleavage rate for the fastest cleaving hammerhead ribozymes at pH 6. Based upon these observations, we conclude that the pH-dependent conformational change is the rate-determining step under standard conditions for the hammerhead ribozyme self-cleavage reaction, and that an ionizable 2′-proton at cleavage site is required for this conformational change. We further hypothesize that deprotonation of the cleavage-site 2′-oxygen drives this conformational change.

Keywords: hammerhead ribozyme; conformational change; mechanism of catalysis; pH-dependence; catalytic RNA

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Introduction

The hammerhead ribozyme[1–5] (Figure 1) catalyzes a self-cleavage reaction that is initiated by nucleophilic attack of the 2′-oxygen atom of the cleavage-site ribose upon the adjacent scissile phos-

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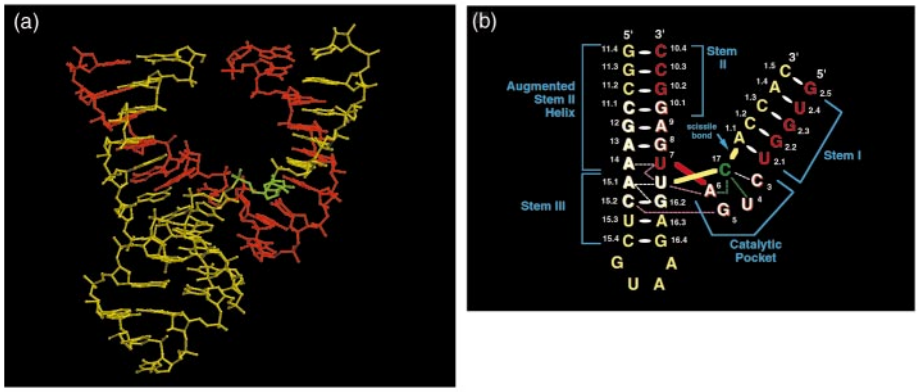

Figure 1. Crystal structure of the initial-state of the hammerhead ribozyme. The enzyme strand is shown in red, the substrate strand in yellow, and the cleavage-site nucleotide, C-17, is highlighted in green. The scissile phosphate lies between C-17 and A-1.1 as indicated by the arrow. The canonical numbering scheme for the nucleotides and helices is indicated.

phate, resulting in formation of a 2′,3′-cyclic phosphodiester and concomitant cleavage of the phosphate backbone of the substrate RNA (Figure 2). The hammerhead ribozyme appears to require divalent cations (Mg^{2+} or Mn^{2+}, Co^{2+}, Cd^{2+}, etc.) for folding or catalysis at low ionic strength,[6,7] but actually does not require metal ions for catalysis at higher ionic strength where the RNA can fold in the absence of divalent cations.[8–10] The hammerhead-catalyzed cleavage reaction is also pH-dependent:

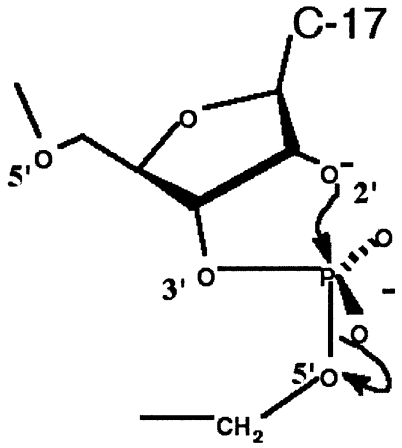

Figure 2. The hammerhead cleavage reaction mechanism involves in-line attack of the 2′-oxygen atom upon the adjacent scissile phosphate. The 5′-oxygen atom, the leaving group, must also be in line with the phosphorus atom and the 2′-oxygen atom. The 2′ and 5′ oxygen atoms therefore occupy the axial positions of a pentacoordinated oxyphosphorane transition-state or intermediate, and the 3′-oxygen atom and the two nonbridging phosphate oxygen atoms occupy the axial positions.

$$\frac{d(\log k_2)}{d(pH)} \approx +0.7$$

between a pH range of 5.5 and about 8.0 to 8.5, depending upon the cation present, but the pH-rate profile plateaus at higher pH values.[7] Based on this behavior, a kinetic pK_a of about 8 in the presence of Co^{2+} and of about 8.5 in the presence of Mg^{2+} can be estimated,[7] and it appears from the log-linear dependence of rate upon pH that a single proton is abstracted in the rate-limiting step of the hammerhead ribozyme cleavage reaction.[7] This observation is generally cited as evidence that the chemical step is rate-limiting in kinetically "well-behaved" hammerhead ribozyme sequences.[11] Typical cleavage rates of kinetically well-behaved hammerhead ribozymes are on the order of 1.0/minute at pH 7.5 in 10 mM $MgCl_2$, although one sequence has been found to cleave up to ten times faster in these conditions.[11–13] This represents up to a 10^6-fold rate enhancement over uncatalyzed, non-sequence-specific RNA self-cleavage.

Crystal structures of hammerhead ribozymes have been obtained in several states, including the initial-state conformation,[14,15] two conformational changes that appear to occur prior to cleavage,[16,17] and in the final or cleaved state.[18] In addition, the pH-dependence of these conformational changes has been assayed crystallographically, and it has been found that these conformational changes do not accumulate to high occupancy in the crystal much below a pH of 8.5.[16,17] We report here that if the 2′-OH group at the cleavage site is replaced by either a 2′-OCH_3 moiety or a 2′-F moiety, thus preventing ionization of the 2′-oxygen atom, the second and more pronounced conformational change cannot be induced, even above pH 8.5.

The hammerhead ribozyme sequences optimized for crystallization cleave more slowly under stan-

dard reaction conditions in solution[14,"16] than do typical kinetically well-behaved hammerhead sequences. The all-RNA sequence in solution cleaves at about 0.04/minute under ''standard'' reaction conditions, at about 0.08/minute in a solution of 1.8 M Li_2SO_4 at pH 8.5, whether or not divalent cations are present, and cleaves at about 0.4/minute under the latter conditions when crystallized and in the presence of divalent cation.[16,17] The hammerhead ribozyme sequence we crystallized has a significant kinetic defect. (Our particular sequence was selected to optimize crystal diffraction properties rather than kinetic properties.) The enzyme strand tends to dimerize under low ionic strength conditions, thus lowering the concentration of active ribozyme in solution. The apparent rate enhancement in the crystal is, therefore, likely due, at least in part, to the elimination of this alternative, non-productive structure. In this case the crystal lattice is doing more to enhance than to inhibit the self-cleavage reaction.

To better characterize the self-cleavage reaction that occurs in hammerhead ribozyme crystals, and to compare it to what occurs in solution, we have examined crystalline ribozyme catalysis under a variety of conditions. X-ray crystallography reveals that crystals of the unmodified (i.e. cleavage-active) hammerhead ribozyme are about 78 % solvent. It is thus possible to activate cleavage in the crystal by soaking crystals in a vast excess of mother liquor (1.8 M Li_2SO_4, divalent cation solution, and buffer at the appropriate pH). We have observed that the extent of RNA self-cleavage in the crystal is essentially complete, whereas in solution the substrate strand is only cleaved to about 70 % completeness, as is observed for several other, kinetically well-behaved, hammerhead ribozyme sequences.[11] We observe a log-linear dependence of k_2 (as determined over the first 120 minutes) upon pH, with a positive slope of 0.70 in the presence of Mg^{2+} and 0.85 in its absence, in accord with what is observed for this as well as kinetically well-behaved sequences in solution. We have also verified the formation of the expected 2′,3′-cyclic phosphate cleavage product in the crystal. We also find that, in contrast to what is observed in solution, the cleavage rate in the crystal is biphasic. The initial or slower phase is the most protracted at pH 6, and is about two and a half hours in duration. The slower phase corresponds to the rate observed in solution at this pH. Remarkably, the faster phase almost parallels the cleavage rate observed at pH 8 (0.4/minute), a cleavage rate comparable to the fastest-cleaving hammerhead ribozyme sequences at pH 6 under otherwise standard reaction conditions (i.e. 0.5/minute for HHα1, assuming the above-cited log-linear dependence of rate upon pH).

From these observations, we conclude: (a) that the larger conformational change that aligns the 2′-oxygen attacking nucleophile with the scissile phosphate[17] is pH dependent and occurs above the kinetic pK_a for the hammerhead ribozyme cleavage reaction; (b) that the pH-dependent conformational change, rather than the chemical step, is rate-limiting; and (c) that the crystallographically observed conformational change appears to require the presence of an unaltered 2′-OH group at the cleavage-site ribose, and because of this and the observation that the kinetic pK_a of the hammerhead ribozyme cleavage reaction matches the pH at which this conformational change is induced, conclude that it must be all or part of the conformational change relevant to catalysis.

Results and Discussion

The larger cleavage-site conformational change requires an unaltered 2′-hydroxyl group to be present at the cleavage site

We have previously solved structures of the hammerhead ribozyme in four different states that occur upon the cleavage reaction pathway. These include an initial-state hammerhead ribozyme,[15,16] an ''early'' conformational change,[16] a ''later'' and more pronounced conformational change that brings the attacking nucleophile, the 2′-oxygen atom of the cleavage-site ribose, into a position close to that required for an in-line attack on the scissile phosphate,[17] and the hammerhead ribozyme cleavage product.[18] The first of these structures contained a 2′-OCH_3 modification of the cleavage site ribose, preventing nucleophilic attack.[15] The other initial-state structure and early intermediate structure[16] were of RNA having an unaltered cleavage-site ribose. The later intermediate structure was one in which the attacking nucleophile was unaltered but the 5′-carbon atom adjacent to the 5′-oxygen atom of the leaving group was altered with a *talo*-methylation, creating a kinetic bottleneck at the bond scission step of the reaction. Both of these conformational changes can only be observed crystallographically subsequent to soaking the crystals in a cleavage-inducing artificial mother liquor buffered to pH 8.5. Below pH 8, the conformational changes cannot be detected, and under these conditions the 5′-modified hammerhead RNA structure[17] is identical with that of the unmodified hammerhead RNA.[16] This lower-pH structure in fact served as an important control that verified the presence of the 5′-*talo*-methyl-modification itself did not induce the altered conformation observed in the later intermediate structure, but rather that the conformational change was a consequence of raising the pH of the artificial mother liquor in the presence of Co^{2+}.

We have now repeated these experiments under identical conditions at pH 8.5 using two different modified hammerhead ribozyme substrates wherein the attacking nucleophile has been altered. One modified hammerhead RNA has a 2′-OCH_3 at the active site, as was used previously to determine the original all-RNA structure,[15] and the other has a 2′-F at the active site, which is isoelectronic to a deprotonated 2′-oxygen atom, but is uncharged.

Although the early conformational change (URX058 in the Nucleic Acid Database) is reproduced almost exactly with both modified RNAs (within estimates of mean coordinate error, Figure 3(a) and (b)), the later, more pronounced conformational change (URX071) that brings the attacking nucleophile into alignment with the scissile phosphate (Figure 3(c) and (d)) does not take place, even after five hours of soaking at pH 8.5 in 50 mM Co^{2+}, 1.8 M Li_2SO_4 and 50 mM Tris (and was repeated) with the *talo*-5′-C-methylated substrate.

The crystals having the 2′-OCH_3 modification diffracted to 2.7 Å resolution and the crystals having the 2′-F modification diffracted to 3.1 Å resolution. Both were refined (independently) using the initial-state structure (URX057) as a starting model for refinement to avoid model bias. Details of data collection and refinement are listed in Table 1, and superpositions of each of the crystal structures with that of URX057, the early conformational intermediate are shown in Figure 3(a) and (b).

The extent of RNA self-cleavage in the crystal is essentially complete

We further characterized the cleavage reaction in the crystal by assaying the extent of cleavage as a function of time (Figure 4(a)). Site-specific cleavage of the RNA substrate proceeds to completion in the crystal, whereas in solution, as with many other hammerhead RNA sequences, it does not. In solution, the reaction plateaus at approximately 70 % of substrate consumed. It is generally assumed that a fraction of the substrate RNA in solution remains uncleaved because it is either kinetically trapped in an alternate conformation that is unrecognized by the enzyme, or because it contains sequence errors or is otherwise chemically non-identical to the active substrate.[11] Because cleavage of the RNA-substrate complex in the crystal must be single-turnover, it is clear that all of the hammerhead ribozyme RNA molecules in the crystal lattice must be able to access the active conformation, and that the substrate must be prevented from forming inactive conformations within the crystal lattice. The hammerhead ribozyme fold, confined within the crystal lattice, must therefore be compatible with the catalytically active conformation of the RNA. When combined with our previous observation that the particular sequence of hammerhead ribozyme that we crystallized cleaves more rapidly in the crystal than in solution under otherwise identical conditions,[17] it is not only clear that the crystal lattice contacts (Figure 4(b)) are doing more to assist than to hinder catalysis, but it makes the argument that the initial-state crystal structure must be reasonably close to the catalytically relevant conformation additionally compelling.

The cleavage reaction in the crystal obeys the expected log-linear relation between k_2 and pH

In solution, the log of the rate-limiting step (characterized by the rate constant k_2) of the hammerhead-catalyzed cleavage reaction is linearly dependent upon pH between a pH range of 5.5 and about 8.0 to 8.5, depending upon the cation present.[7] In the presence of Mg^{2+} under standard conditions, the slope of the line is 0.7, indicating that one proton is transferred during the rate-limiting step of the reaction, but its dissociation in the transition-state may only be partial. We assayed the rate of hammerhead ribozyme self-cleavage in the crystal as a function of pH and found that in the presence of Mg^{2+}, the slope again is 0.7, but in the absence of Mg^{2+} it increases to 0.85 (Figure 5). The former result indicates that the same rate-limiting step measured in solution is the rate-limiting step in the crystal in the presence of Mg^{2+}. The latter result indicates that in the absence of Mg^{2+} the details of the reaction differ slightly in the sense that there appears to be marginally greater proton dissociation in the transition-state of the rate-limiting step of the reaction, but overall that the same pH-dependent step is rate limiting.

The cleavage rate in the crystal is biphasic

We have also observed that, in contrast to what takes place in solution, the cleavage rate for the hammerhead ribozyme in the crystal is biphasic (Figure 6). A pH-dependent lag or slower cleavage phase followed by an almost pH-independent rapid-cleavage phase is observable in the cleavage rate profiles in the crystal for all cases measured. This effect is most prominent at lower pH. At pH 6, the slow phase is about 150 minutes in duration, and it corresponds to the rate observed in solution at this pH. Subsequently, the rate of cleavage in the crystal increases to a rate almost paralleling the cleavage rate observed at pH 8 (0.4/minute).

The rapidly cleaving phase at pH 6 is comparable to the fastest-cleaving hammerhead ribozymes

Remarkably, in the case of the rapidly cleaving phase at pH 6.0 in the hammerhead ribozyme crystals, the rate of cleavage in the crystal increases to a rate almost paralleling the cleavage rate observed at pH 8.0 (0.4/minute). This is a cleavage rate comparable to the fastest cleaving hammerhead ribozyme sequences at pH 6.0 under otherwise standard reaction conditions (i.e. 0.5/minute for HHα1, assuming the above-cited log-linear dependence of rate upon pH. The cleavage rate in 1.8 M Li_2SO_4 might be further reduced by as much as tenfold, based upon our measurements with HH16.1, (*cf* reference 17), meaning that the cleavage rate of 0.4/minute in the rapid phase might more justifiably be compared to a HHα1 solution cleavage rate of about 0.05/minute under these

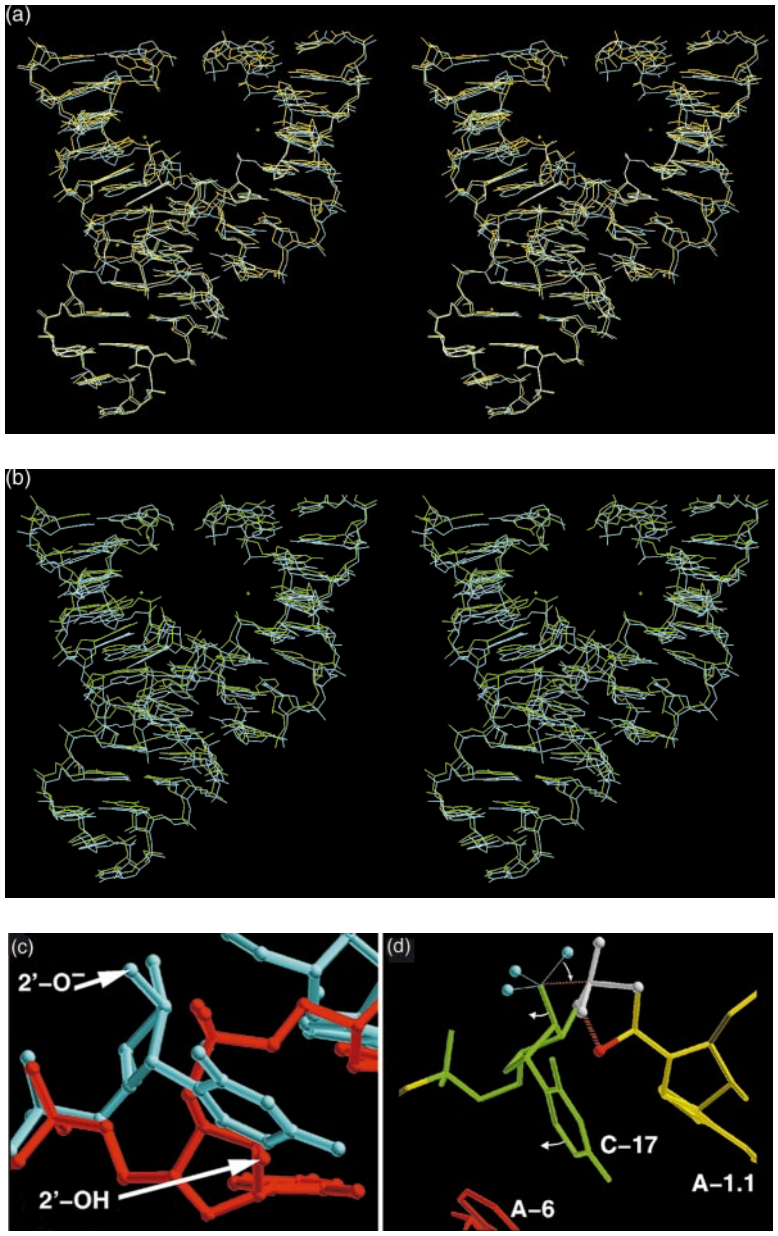

Figure 3. (a) Stereo superposition of the 2′-OMe-modified C-17 hammerhead ribozyme (shown in yellow) upon the unmodified 2′-OH ''early'' intermediate structure (shown in cyan), showing the near-identity of the two structures. The r.m.s.d for all atoms is 0.525 Å, and for the scissile phosphate atoms of A-1.1 and attacking ribose of C-17 is 0.493 Å. (b) Stereo superposition of the 2′-F-modified C-17 hammerhead ribozyme (shown in green) upon the unmodified 2′-OH ''early'' intermediate structure (shown in cyan), again showing the two are very similar. The r.m.s.d for all atoms is 0.568 Å, and for the scissile phosphate atoms of A-1.1 and attacking ribose of C-17 is 1.046 Å. (c) The initial-state (red) and ''later'' conformational intermediate (blue) hammerhead ribozyme cleavage site nucleotide (C-17). The scissile phosphate of the intermediate structure is here omitted to avoid placing restraints upon the position of C-17 during refinement, as described.[8] (d) Formation of a trigonal bipyramidal oxyphosphorane transition-state in the ''later'' conformational intermediate structure is apparently hindered by a steric clash (indicated by the thick red broken line) between the *talo*-5′-methyl modification (red atom) of the A-1.1. ribose and an oxygen atom of the scissile phosphate (shown in white). Alignment of one of the electron lone pairs on the attacking oxygen nucleophile (represented as blue spheres on the 2′-oxygen atom) to form the transition-state in the absence of the extra methyl group would then require small additional movements of the C-17 base and ribose (green) as indicated by the small curved white arrows.

conditions. Since the slower-cleaving phase (or more properly, the ''normal'' cleaving phase) is much more pronounced at pH 6 than at pH 7 or 8, it is unlikely that either the rate of diffusion of the artificial mother liquor containing divalent cations and buffer, or the time taken for the crystal to equilibrate, are the primary causes for these ''lags''. A more likely explanation is that the conformational

Table 1. Author to supply short title

Experiment	2′-OMe modified C-17 50 mM $CoCl_2$ 5 hour soak at pH 8.5	2′-F modified C-17 50 mM $CoCl_2$ 5 hour soak at pH 8.5
A. *Data collection*		
X-ray source	Brookhaven X-12C	Brookhaven X-12C
Wavelength (Å)	1.1	1.1
Resolution (Å)		
Overall	18.8 to 2.7	19.9 to 3.1
High res. bin	2.85-2.7	3.27-3.1
Completeness (%)		
Overall	97.3	90.8
High res. bin	90.1	92.4
$I/\sigma(I)$		
Overall	33.4	24.8
High res. bin	2.8	1.2
R_{sym}/R_{scale}		
Overall	0.059/0.076	0.096/0.113
High res. bin	0.269/0.356	0.491/0.674
Cell (*a*, *c* in Å)	65.74, 138.0	65.76, 139.23
B. *Refinement*		
R-factor ($F > 2\sigma$)	0.257	0.248
R_{free} (10 % data)	0.323	0.290
Geometry		
r.m.s.d. bonds (Å)	0.014	0.011
r.m.s.d. angles (°)	1.89	1.68
r.m.s.d. torsion angles (°)	18.55	19.06
r.m.s.d. planarity (°)	2.1	2.0

change observed in the crystal is pH-dependent, and that once this conformational change has taken place, cleavage proceeds rapidly in an almost pH-independent manner. At pH 6, which is about 2 to 2.5 units below the kinetic pK_a and the apparent pK_a of the conformational change, only a small fraction of the molecules in the crystal will visit the changed conformation at any particular time. When these molecules do so and cleave, they will then occupy a new conformation[18] that may then help to induce the required conformational change cooperatively in the neighboring molecules through crystal lattice contacts. Once a critical fraction of the RNA has cleaved, the cooperative transition takes place throughout the crystal lattice, and the RNA then becomes conformationally equivalent to the most kinetically competent hammerhead ribozyme sequences, and correspondingly rapid cleavage takes place.

Conclusions

The conformational change may be the rate-limiting step in hammerhead ribozyme catalysis

The log-linear dependence of the cleavage rate upon pH is generally accepted as *prima facia* evidence that the chemical step is rate limiting in kinetically well-behaved hammerhead ribozyme sequences. The kinetic pK_a of the reaction is reported to be about 8.5 in the presence of Mg^{2+} and about 8.0 in the presence of Co^{2+}. We have observed experimentally a conformational change that appears to align the attacking nucleophile with the scissile phosphate.[17] This conformational transition cannot be induced below approximately pH 8.5, indicating that the kinetic pK_a and the conformational transition pK_a are the same within the accuracy of crystallographic titration (±0.5), and quite likely correspond to the same physical phenomenon. In addition, the biphasic nature of the cleavage reaction in the crystal fortuitously enables us to isolate and measure the rapid-cleaving phase and to demonstrate that it appears to be approximately pH-independent. It therefore appears that $k_2 \neq k_{cat}$, but instead that the following kinetic scheme for the forward reaction holds:

$$E + S \xrightarrow{k_{+1}} ES \xrightarrow{k_{+2}} ES^* \xrightarrow{k_{cat}} E + P$$

where E and S have their usual meaning, k_2 is the pH-dependent rate-limiting step, and k_{cat} is the chemical step and is more rapid than k_2 under standard reaction conditions in solution. ES represents the initial-state hammerhead ribozyme structure, and ES* represents the conformationally changed structure poised for catalysis. Given the similarity between (and probable identify of) the observed kinetic and structural pK_a values, we equate the crystallographically trapped ''later'' conformational intermediate structure[17] with all or a significant, rate-limiting subset of the structural changes required for formation of the cleavage-active structure. It is perhaps noteworthy that the vexing myriad of nucleotide alterations in the hammerhead ribozyme that have been found to alter k_2 but not substrate binding and are nevertheless located far from the cleavage site in the crystal structure (reviewed comprehensively in reference 19) can then be understood as mutations that inhibit the conformational change required for catalysis, rather than mutations that disrupt the chemical interactions required in the actual transition-state of the cleavage reaction.

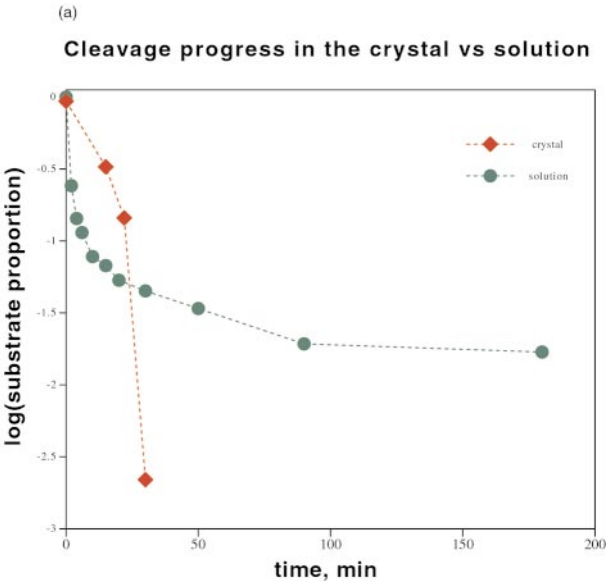

Figure 4. (a) Extent of site-specific cleavage at C-17 of the hammerhead RNA sequence at pH 7.4 shown in Figure 1 in solution (green) and in the crystal (red). Only about 70 % of the substrate RNA is cleaved in solution, whereas the cleavage reaction goes to completion in the crystal. (b) Lattice contacts in the hammerhead ribozyme crystal constrain stem I *via* 5′ to 5′ and 3′ to 3′ stacking interactions with stem II of a neighboring molecule (pink) as well as *via* contacts between stem I and loop III of another neighboring molecule (yellow). These lattice contacts may account for the apparent cooperative nature of the biphasic cleavage rate profile.

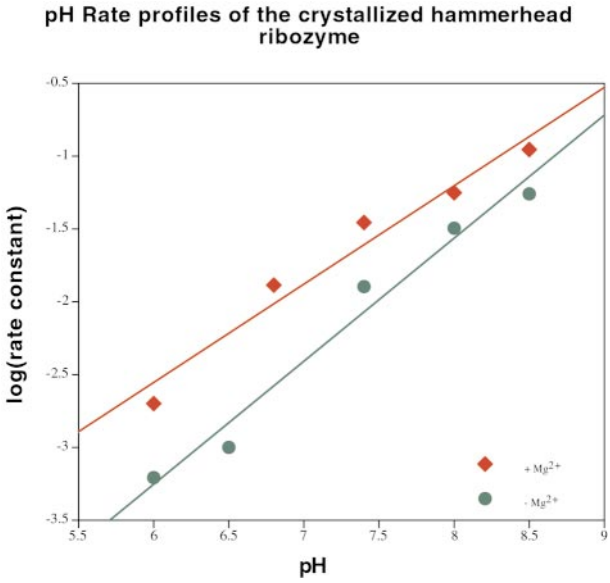

Figure 5. Hammerhead ribozyme cleavage rates in the crystal as a function of pH. In crystals soaked with an artificial mother liquor having 1.8 M Li_2SO_4, both in the presence and in the absence of Mg^{2+}, the cleavage rate is approximately log-linear with respect to pH between pH 6 and pH 8.5. The lines have slopes of 0.7 and 0.85 in the presence and absence of Mg^{2+}, respectively. Figure 2 from Dahm *et al.*[7] shows essentially the same behavior in the presence of Mg^{2+} for the hammerhead ribozyme in solution. Dahm *et al.*[8] estimate an apparent pKa of about 8.5 in the presence of Mg^{2+} and an apparent pK_a of about 8 in the presence of Co^{2+} can be estimated, based on Figure 5.

The proton of the active site 2′-hydroxyl group is required for the observed conformational change

It is well known that the 2′-proton is abstracted from the cleavage-site nucleophile prior to or during hammerhead ribozyme catalysis. Our finding that either a 2′-OCH_3 or a 2′-F substitution at the cleavage site inhibits cleavage (as expected) as well as preventing the ''later'' conformational change from occurring, entails that an unaltered active-site 2′-hydroxyl group is required for the conformational change to take place. Because the initial-state and ''early'' conformationally changed structures are unperturbed by the presence of either substitution, and the fact that these substitutions would be sterically unhindered and capable of free rotation in the ''later'' conformationally changed structure, and finally the electronic properties of the unionized and ionized 2′-oxygen atom are preserved by the 2′-OCH_3 and 2′-F substitutions, respectively, it is clear that the 2′-proton must play a critical role in driving or stabilizing the conformational change. The two most obvious possibilities are that this proton forms a hydrogen bond that stabilizes the conformationally-changed structure or that this proton is abstracted from the 2′-oxygen atom attacking nucleophile, thus driving the transition to, or perhaps helping to stabilize, the pre-catalytic conformational intermediate structure. However, no such hydrogen bond can be inferred from the crystal structure of the conformational intermediate, and hydrogen-bond formation does not account for the log-linear

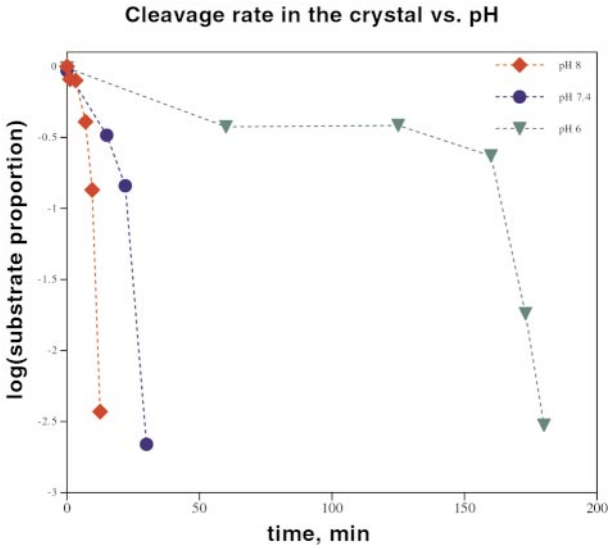

Figure 6. Cleavage rate of the hammerhead ribozyme in the crystal *versus*. pH in the presence of Mg^{2+}. The rate of the cleavage reaction is seen to be biphasic, with the initial-phase being strongly pH-dependent, and the final phase being almost pH independent. The initial-phase was most pronounced at pH 6, and was became negligible above pH 8.

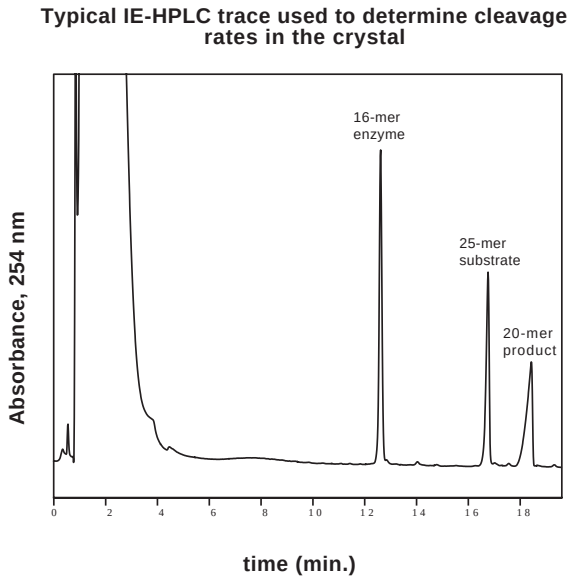

Figure 7. Typical ion-exchange HPLC chromatogram used to assay a single crystal for a single timepoint in a cleavage kinetics assay. The enzyme peak (Rz) corresponds to the 16-mer enzyme strand (see Figure 1) and the substrate (Sub) and product (Prod) strands correspond to 25-mer and 20-mer strands, respectively. The 2′,3′-cyclic phosphate appears to be responsible for the anomalous retention time for the 20-mer product strand. The 5-mer product strand was not separable from the large breakthough peak under these conditions. The breakthrough peak was assayed and found to be composed primarily of Tris or other buffers, EDTA, glycerol, salts and other components of the artificial mother liquor, and did not contain RNA apart from traces of the 5-mer.

dependence of the rate-limiting step upon the pH or the implied proton transfer. Ionization accounts for the observed pH-dependence and could well drive or stabilize the conformational change; however, this explanation suffers from the disadvantage that the kinetic pK_a (and conformational pK_a) is about 8.5 and the pK_a for ionization of this proton from a ribose in solution is about 12 to 13. A mechanism by which this pK_a might be perturbed significantly by interactions between the ribose and the catalytic pocket nucleotides has, however, been proposed.[5]

Experimental Methods

Assay of cleavage activity in the crystal

The cleavage activity in the crystalline state was initiated by immersing hammerhead crystals in an artificial mother liquor solution at the specified pH containing 1.8 M Li_2SO_4, 20 % (w/v) glycerol (used originally as a cryoprotectant and to aid stabilization of the crystal lattice), 10 or 50 mM Co^{2+} or Mg^{2+} and 50 mM of the appropriate Tris buffer. The cleavage reactions were then quenched by removing crystals from the mother liquor solution with a rayon loop (used for cryocrystallography) and immediately immersing it in 500 mM EDTA. The crystals were then dissolved in approximately 3 ml of water immediately prior to IE-HPLC analysis[20] (Figure 7). The sample was then analyzed at 50 °C by ion-exchange HPLC, (Dionex DNA-PAC) using a gradient of 350 mM to 650 mM NH_4Cl over 30 minutes. A representative chromatogram is shown in Figure 7. Controls containing either 0 or 100 % cleaved RNA were used to identify the locations of the substrate and product peaks. The extent of cleavage was determined from the ratios of enzyme strand to substrate, enzyme strand to product and product to substrate. Each method of calculation gave values that agreed to within ±5 %. The enzyme, substrate and product strands were identified by co-injection with the appropriate standard. Individual time points for rate analyses were assayed using two crystals; i.e. each single-turnover measurement is the average of a duplicate experiment. Between four and ten (most typically six) time points were recorded for each cleavage-rate assay, and each individual measurement was obtained from a single crystal measuring about 0.15-0.2 mm × 0.15-0.2 mm × 0.3-0.5 mm. The crystallized RNA guaranteed that all reactions were single-turnover by virtue of the fact that the RNA crystallized only as a 1:1 stoichiometric complex between enzyme and substrate.

Crystallization

Crystals containing hammerhead ribozyme substrates having either a 2′-OMe modification or a 2′-F modification of C-17, as well as the wild-type (2′-OH C17) RNA were grown using crystallization conditions as reported, i.e. with 1 mM ribozyme in 50 mM NaOAc

buffer, pH 5.0, 1.8 M Li_2SO_4 in the absence of Mg^{2+} and other divalent cations. The various strands of RNA were synthesized using oligoribonucleotide phosphoramidite chemistry, using deoxycytosine solid-phase supports. The RNA was purified successively by anion-exchange HPLC and C-18 reverse-phase HPLC and subsequently de-salted. Four microliters of the RNA solution were then combined with 2 µl of reservoir solution (50 mM NaOAc, pH 5.0, 1.8 M Li_2SO_4, and 1.0 mM EDTA), and equilibrated as hanging or sitting drops against 0.75 ml of the reservoir solution sealed in a Linbro tissue culture plate at 16 °C. The best crystals (0.2 mm × 0.2 mm × 0.3 mm) grew in these initially 6 µl drops rather than larger drops, formed within two to three days.

Collection of X-ray diffraction data

Each crystal was soaked in a freezing solution consisting of 20% glycerol, 50 mM Tris buffered at pH 8.5, 1.8 M Li_2SO_4, and 50 mM $CoCl_2$ for at least 300 minutes. In both cases the soaking experiments were terminated by flash-freezing the crystals in liquid propane cooled in a bath of liquid nitrogen. Further details of data collection are described in Table 1. We assayed the cleavage in the crystal by HPLC as described[17] and detected no cleavage, consistent with the modifications at the cleavage site.

Crystallographic refinement

Initial rigid-body refinement followed by conventional positional refinement (Powell minimization) in X-PLOR 3.8[21] was performed to refine a starting model (URX057) for each RNA crystal structure. This starting model was then further refined using a standard simulated annealing slow-cooling molecular dynamics protocol followed by conventional positional and (highly) restrained temperature factor refinement in X-PLOR 3.8 using data from 8.0 Å to 3.0 Å resolution and a modified RNA geometry parameter library.[22] This procedure lowered the *R*-factor to about 25% in each case while maintaining very precise geometry during the final refinement, the statistics of which are reported in Table 1.

Protein Data Bank accession code

The coordinates have been deposited in the RCSB Protein Data Bank, with accession code 301D.

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