

Ribozyme Catalysis *via* Orbital Steering

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Orbital steering is invoked to explain how the three-dimensional structure of a small self-cleaving RNA, the hammerhead ribozyme, both prevents and enhances RNA autocatalysis. Within the conserved catalytic core of the ribozyme, the position of the 2' oxygen atom of the G8 ribose is observed to be aligned almost perfectly with the phosphorus atom and the 5' oxygen atom of the adjacent A9 phosphate group for self-cleavage *via* an in-line attack mechanism. Despite this apparent near-perfect atomic positioning, no cleavage takes place. The explanation proposed is that a network of hydrogen bonds in the ribozyme core orients or steers the orbitals containing the electron lone pairs of the attacking nucleophile (the 2' oxygen atom) away from the A9 phosphorus atom, eliminating overlap with the vacant phosphorus d-orbitals despite the near-perfect in-line positioning of the oxygen atom, thus preventing catalysis. Because of the near-perfect atomic positioning of the 2' oxygen atom relative to the phosphate group, orbital steering effects in this case are fortuitously uncoupled from conformational, distance and orientation effects, allowing an assessment of the catalytic power due purely to orbital steering. In contrast, a conformational change at the cleavage site is required to bring the 2' oxygen atom and the scissile phosphate group into atomic positions amenable to an in-line attack mechanism. In addition, the conformationally changed structure must then steer the lone-pair orbitals of the correctly positioned 2' oxygen atom toward the scissile phosphorus atom in order for cleavage to take place. We estimate that fulfilment of each of these two required changes may contribute separately an approximately 1000-fold rate enhancement, potentially accounting for a significant fraction of the catalytic power of this ribozyme. Orbital steering therefore appears to be a general phenomenon that may help to explain catalysis in both ribozymes and protein enzymes in a unified manner.

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Introduction

The simplest ribozyme-catalyzed chemical reaction is self-cleavage of the phosphodiester backbone. This reaction takes place *via* a process of phosphodiester isomerization in which a 2' proton is abstracted from a ribose, and the resulting nucleophilic attack of the ionized 2' oxygen atom upon the adjacent phosphate group results in a break of the phosphate backbone in which the product RNA strands have 2',3'-cyclic phosphate and 5' hydroxyl termini at the site of cleavage.¹ This reaction is catalyzed by four of the eight known natural ribozymes, i.e. the hammerhead, hairpin,

HDV and VS self-cleaving RNAs. In many respects the reaction is identical with the random, base-mediated degradation of RNA that accounts for its inherent instability. The main differences are that the ribozyme-catalyzed reactions are completely sequence-specific and occur at a rate enhanced approximately 100,000-fold compared to the basal rate of RNA self-cleavage. Both the catalyzed and uncatalyzed reactions take place *via* an $S_N2(P)$ mechanism in which the attacking nucleophile, the ionized 2' oxygen atom, must be positioned in line with the phosphorus atom and the leaving group 5' oxygen atoms, such that the required pentacoordinated oxyphosphorane transition-state structure is that of a trigonal bipyramid in which the 2' and 5' oxygen atoms occupy the axial positions.¹

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The hammerhead ribozyme²⁻⁴ is particularly well-suited to biophysical studies because of its small size, well-characterized biochemistry, biological relevance, and its known crystal structures determined at several points along the reaction pathway. The initial-state crystal structure of the hammerhead ribozyme,⁵⁻⁷ (Figure 1(a) and (b)), is particularly intriguing, because the scissile phosphate group is maintained in the geometry ($g - ,g-$) found in A-form RNA helices (Figure 2(a) and (c)), whereas another phosphate group, that at position 9, is found to be in a rather different geometry, one that is almost perfectly compatible with an in-line attack mechanism (Figure 2(b) and (d)). An "in-line fitness parameter" has been devised,⁸ whereby a perfectly aligned phosphate group, having the 2' oxygen atom positioned 3.0 Å from the phosphorus atom, is assigned a geometrical fitness value of 1.0, and the most poorly aligned phosphate group (one in which the attacking and leaving oxygen atoms would be positioned 90° from one another in a trigonal bipyramidal transition-state), is assigned a fitness value of 0.0. Using this characterization of in-line fitness, the scissile phosphate group found in the initial-state crystal structure of the hammerhead ribozyme has a fitness parameter of 0.05, and that at position 9 has a value slightly greater than 1.0. Cleavage at the scissile phosphate group, based upon its observed geometry, would then be predicted to occur at a rate of about 10⁻⁸ per minute, whereas cleavage at the position 9 phosphate group would be predicted to occur at about 10⁻³ per minute, given its conformation. Despite these observations and predictions, actual cleavage at the scissile phosphate group, in the crystal, has been measured to occur on the order of 0.4 per minute, and cleavage at the position 9 phosphate group is undetectable, both in the crystal and in solution. In other words, the reactivity of the two phosphate groups is exactly the opposite of what one might reasonably predict, based solely upon their respective geometrical configurations.

Why is it that no cleavage occurs at the phosphate group at position 9, despite the fact that it is aligned almost perfectly for an in-line attack? Similarly, why does cleavage occur at the scissile phosphate group despite the fact that it is geometrically completely unfit for an in-line attack? Clearly, some factor, in addition to proximity and geometric "fitness", must determine the potential reactivity of these phosphate groups. By elucidating the nature of the factor responsible for preventing catalysis of a perfectly aligned phosphate group (i.e. one capable of a three to five orders of magnitude suppression of catalysis), it might thus be possible to illuminate the contribution of this factor to the observed 100,000-fold cleavage rate enhancement catalyzed by the hammerhead ribozyme. Fortuitously, the hammerhead ribozyme has this peculiar anti-symmetric relationship between the geometries and cleavage activities at these two phosphate groups, thereby providing an internal control to test the validity of any mechanism proposed to describe ribozyme-mediated catalytic cleavage of the phosphodiester backbone of RNA.

Based upon the initial-state structure of the hammerhead ribozyme,⁷ as well as those of recently observed pre-catalytic ribozyme conformational intermediates^{7,9} and an enzyme-product structure,¹⁰ we propose an "orbital steering" mechanism¹¹⁻¹³ to explain both the suppression of self cleavage at phosphate 9 and the enhancement of catalysis at the scissile phosphate group. In this analysis, we shall propose that the crucial factor that determines catalytic activity, in addition to atomic position as characterized by "in-line fitness," is the orientation of the orbitals containing the non-bonding (or lone-pair) electrons of the attacking nucleophile. As evidence for this proposal, we will demonstrate that the structure of the hammerhead ribozyme fixes (or steers) the orientation of the electron lone-pair orbitals on the 2' oxygen atom away from the adjacent phosphorus atom at position 9, thus preventing nucleophilic attack from occurring, despite the seemingly favor-

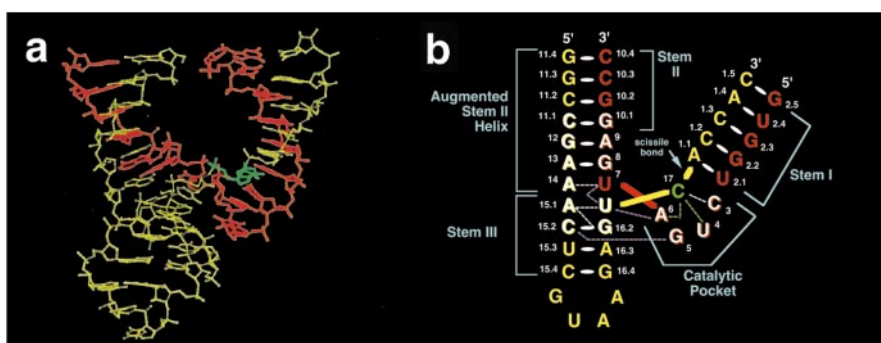


Figure 1. (a) The initial-state crystal structure of an unmodified hammerhead ribozyme.⁷ This particular construct was selected based upon its ability to form crystals suitable for data collection. It consists of a 16 nucleotide enzyme strand (red) and a 25 nucleotide substrate strand (yellow). The cleavage-site nucleotide (C17) is indicated in green, and various regions of the ribozyme, as well as the numbering system used for hammerhead ribozymes, are identified (b) in the corresponding diagram.

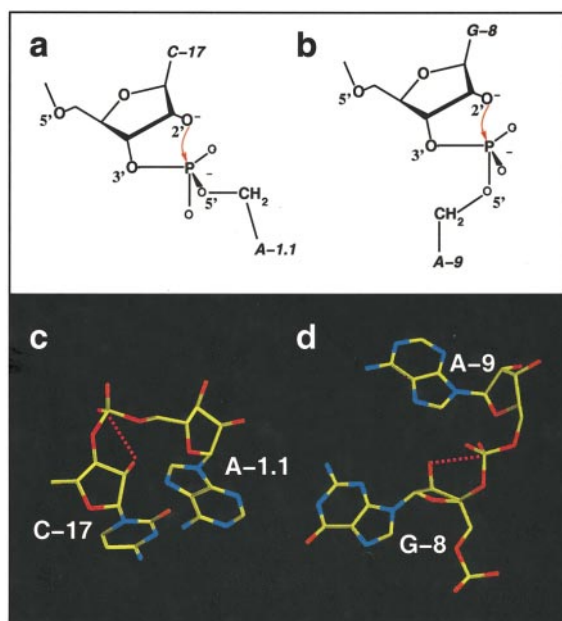


Figure 2. The geometry of the scissile phosphate group between C17 and A1.1 (shown in (c)), is in a conformation ($g-, g-$) that is incompatible with the known in-line attack mechanism. (b) The geometry of the A9 phosphate group (shown in (d)), is almost perfectly arranged for an in-line attack. Cleavage nevertheless occurs at the scissile phosphate group but not at the A9 phosphate group.

able geometry of the phosphate group. Building upon this finding, we show how the attacking 2' oxygen nucleophile adjacent to the cleavage-site phosphate group, as observed in the "late" intermediate structure, can be oriented appropriately *via* continued movement of the cleavage-site base along the trajectory extrapolated between this and the cleavage-product structures, thus allowing catalysis *via* orbital steering to take place.

The A9 phosphate geometry should favor an in-line attack

The structure of the unmodified, and therefore catalytically active, hammerhead ribozyme has been determined both in the absence and presence of divalent metal ions.⁷ In the absence of divalent cations, as well as in the presence of Mn^{2+} and other divalent cations bound to this site,⁵ the phosphate group at position A9 adopts a conformation that positions the 2' oxygen atom of an adjacent nucleotide, G8, in line with the phosphorus atom and the 5' oxygen atom of A9. The structure of the hammerhead ribozyme in the absence of any metal ions is the most reliably refined structure of the completely unmodified hammerhead RNAs.⁷ In this structure (URX057 in NDB⁷), the distance between O2' and P is 2.77 Å, that between O2' and O5' is 4.34 Å, and that between O5' and P is 1.59 Å. The angle of attack is therefore 169.56° and

the in-line fitness, according to the formulation of Soukup & Breaker,⁸ is $F = 1.17$. This value exceeds 1.0 because the distance chosen for the ideal reference is 3.0 Å. Using the composite plot depicting the relationship between in-line fitness and measured rate constant for RNA transesterification for 136 linkages obtained from several RNA structures,⁸ one might reasonably expect to observe a cleavage rate having an approximate magnitude of 10^{-3} /minute, or a four to five orders of magnitude-enhanced cleavage rate relative to an RNA phosphodiester linkage structurally constrained to a conformation in which $F < 0.1$.

Despite the prediction of enhanced susceptibility to cleavage, the A9 phosphate group is observed to be completely stable, both in solution and in the crystal. Although it may be argued that the solution conformation of the A9 phosphate group might be different from that observed in the crystal structure, the fact remains that no RNA cleavage at the A9 phosphate group is detectable at this site in the crystal, where the geometry is known, even after prolonged incubation of crystals with Mn^{2+} or Mg^{2+} . By contrast, pH-dependent cleavage rates at the scissile phosphate group, as low as 10^{-6} , are detectable using an assay developed explicitly for detecting RNA cleavage in the crystal.¹⁴ Therefore, it is clear that the A9 phosphate group is somehow protected from self-cleavage, despite being arranged in a conformation that is highly amenable to in-line attack.

Orbital steering prevents the self-cleavage reaction at the A9 phosphate group

What protects the A9 phosphate group from self-cleavage, despite being positioned almost perfectly for in-line attack, and despite the fact that its non-bridging *pro-R* oxygen atom has been observed in several crystal structures^{5,7} to bind directly to divalent cations (an interaction believed to further enhance catalysis in RNA self-cleavage reactions)? The answer to this question appears when one considers the hydrogen bonding network between G8, A9 and G12, as pictured in Figure 3, which includes six potential hydrogen bonds. These hydrogen bonds include the reversed-Hoogsteen hydrogen bonding between A9 and G12, a hydrogen bond between the exocyclic amine group of G12 (the donor) and O2' of G8 (the acceptor), and a hydrogen bond between the O2' of G8 and the *pro-S* non-bridging phosphate oxygen atom of A9. An additional contact between this phosphate oxygen atom and the C8 hydrogen atom of A9 may be a hydrogen bond of the sort observed in a DNA quadruplex (K. Phillips *et al.*, unpublished data).

The two hydrogen bonds that involve the O2' of G8 appear to be particularly important. The hydrogen atom bound to O2' must be donated to the non-bridging phosphate oxygen atom, as this atom is unprotonated at neutral pH. The O2' of G8 must also be the acceptor of the hydrogen bond donated

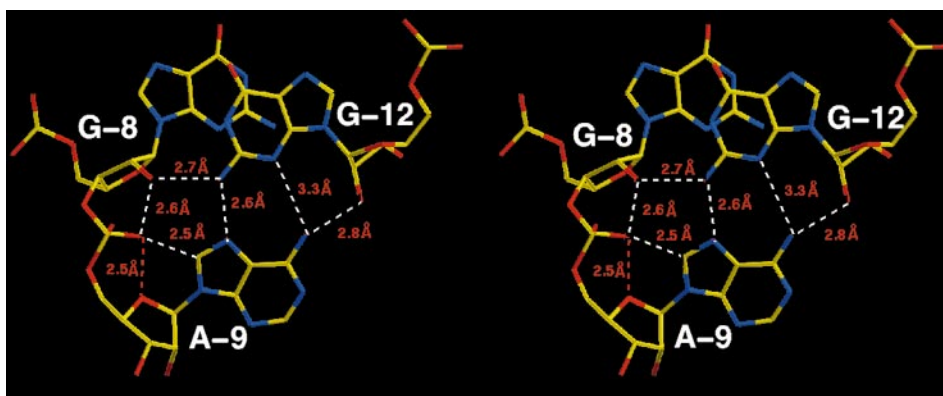


Figure 3. The hydrogen bonding network involving G8, A9 and G12 is extensive, and includes two hydrogen bonds that involve the O2' of G8.

by the exocyclic amine group of G12. Assuming approximate sp^3 hybridization of the O2' valence atomic orbitals, the hydrogen atom and two sets of electron lone-pairs together form an approximately tetrahedral configuration, with C2', H2' and the two non-bonding orbitals containing the electron pairs each occupying the vertices of the tetrahedron. This being so, the two hydrogen bonds, by necessity, orient the tetrahedron, and therefore fix the location of both lone-pair orbitals in space relative to the rest of the RNA structure. One of the two sets of lone pairs forms the hydrogen bond to G8, and the other does not interact directly with the RNA structure. Each of the two hydrogen bonds independently steers both of the lone-pair orbitals away from the phosphorus center, thereby preventing nucleophilic attack and bond formation. The lone-pair orbital not involved in the hydrogen bond is steered 90° away from the phosphorus

atom, thereby ensuring that this orbital is orthogonal to the unoccupied d-orbitals of the phosphorus atom. (The trigonal bipyramidal configuration that the phosphate group adopts in the oxyphosphorane transition state or intermediate structure requires dsp^3 hybridization of the phosphorus valence orbitals.) The other lone pair is sequestered as a hydrogen bond acceptor with the exocyclic amine group of G12, and is oriented about 120° away from the phosphorus atom (Figure 4). The hammerhead ribozyme thus uses a hydrogen bonding network in the vicinity of the A9 phosphate group to orient the O2' non-bonding orbitals, and therefore the potentially reactive electron lone pairs, in a doubly redundant manner that prevents nucleophilic attack upon the A9 phosphate group. Orbital steering, therefore, is employed by the RNA structure to inhibit aberrant self-cleavage by lowering the reactivity of this

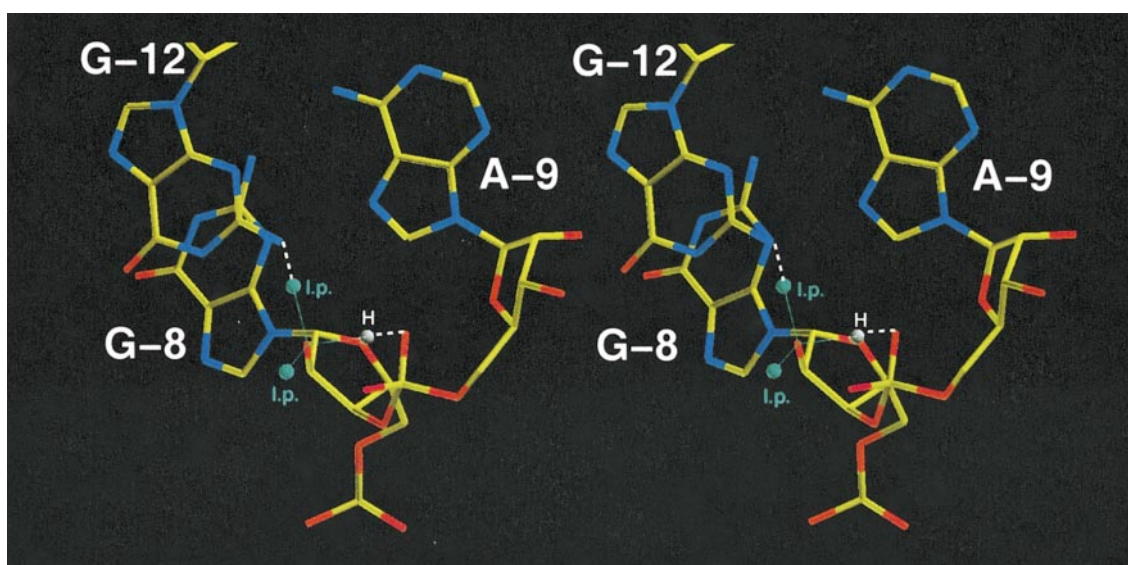


Figure 4. One of the O2' hydrogen bonds on G8 involves hydrogen donation to the *pro-S* exocyclic phosphate oxygen atom of A9, and the other involves hydrogen acceptance from the exocyclic amine group of G12. Each of these hydrogen bonds by itself is sufficient to "steer" the lone-pair electrons (indicated as light-blue spheres labeled l.p.) away from the scissile phosphorus atom, thereby preventing orbital overlap and therefore bond formation. These hydrogen bonds together thus inhibit RNA cleavage in a doubly redundant manner.

phosphate group by at least three orders of magnitude. This is accomplished simply by steering the orbitals away from the direction required for a nucleophilic attack, despite the fact that the attacking and leaving group oxygen atoms are aligned almost perfectly with respect to the phosphorus atom for an in-line nucleophilic attack to occur. We therefore propose that two conditions, rather than just one, must be present for RNA self cleavage to occur. First is the well-known geometrical requirement for in-line attack: the positions of the 2' oxygen atom, the phosphorus atom, and the 5' oxygen atom must all be in-line with one another. The second is that one of the O2' lone-pair orbitals must be oriented in such a manner that significant overlap between it and an unoccupied phosphate d-orbital can occur, once the first condition has been met.

It is noteworthy that binding of Zn^{2+} to the A9 phosphate group appears to enhance the reactivity of this site somewhat, relative to other divalent metal ions (S. Sigurdsson, personal communication). Unlike the other metal ions, Zn^{2+} prefers tetrahedral coordination, and thus distorts the metal binding site in such a way as to begin to steer the nucleophilic lone pair orbital toward the A9 phosphorus atom (W.G.S., unpublished results).

The scissile phosphate group must change conformation to favor in-line attack

The initial-state structure of the unmodified, and therefore catalytically active, hammerhead ribozyme has been determined in both the absence and the presence of divalent metal ions,⁷ and its structure is essentially identical with those in which the cleavage site attacking nucleophile has been altered to prevent catalysis.^{5,6} It has been shown that this

particular unmodified construct can cleave in the crystal at a rate that is somewhat greater than in solution under otherwise identical conditions, i.e. at 0.4/minute in 1.8 M Li_2SO_4 and 25 mM Co^{2+} at $pH \leq 8.5$.⁹ This indicates that the crystallographically observed structures must possess at least some degree of similarity to the catalytically active structure.

Despite this observation, the cleavage-site phosphate group in all of the initial-state structures is in a conformation that is almost completely incompatible with in-line attack (Figure 2(a) and (c)), the (g^- , g^-) phosphate configuration typically observed in A-form helices,^{15,16} and can be characterized with an in-line fitness parameter of $F = 0.05$. It is therefore fairly clear that a conformational change must occur prior to cleavage,⁵ although the nature and extent of the required change has been the subject of some debate.^{7,9,10,17-21}

Conformational and orbital steering requirements for hammerhead ribozyme catalysis

We have previously trapped and observed, using X-ray crystallography, a conformational change that rotates the nucleotide (base and sugar as one rigid body) about the β -angles of the phosphate groups of residues 17 and 1.2 in such a way as to flip the 2' oxygen atom of the cleavage-site nucleotide (C17) toward the phosphate group (Figure 5(a)).²¹ We have also demonstrated that the principal alternative model²⁰ appears to be untenable, at least in its current formulation,²¹ and that the products of hammerhead ribozyme cleavage in the crystal are also observable using X-ray crystallography,¹⁰ indicating that the reaction can take place within the confines of the crystal lattice. For

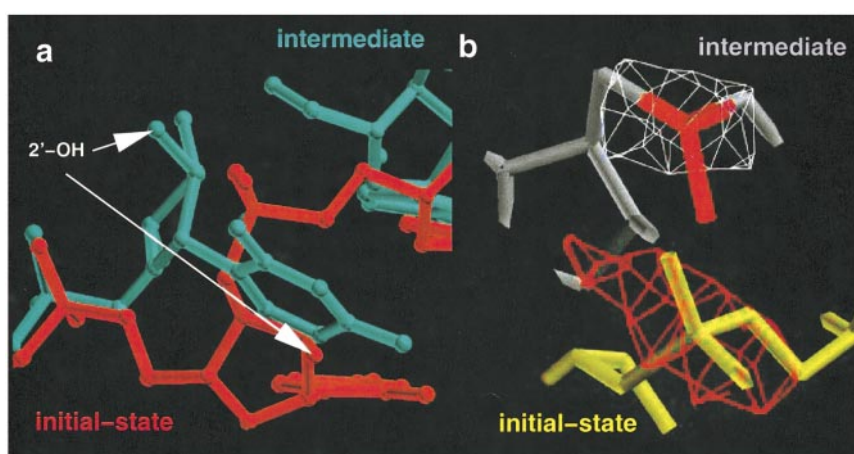


Figure 5. (a) The initial state (red) and conformational intermediate state (blue) configurations of C17, illustrating that the base and ribose move as a rigid body about the β -angle of the phosphodiester backbone in such a way as to reposition the attacking nucleophile, the 2' oxygen atom of C17, nearer to the scissile phosphate group. (b) A difference Fourier peak that corresponds to the center of mass of the phosphate group at 3 Å resolution.⁹ Together, these results illustrate that conformational change has the effect of arranging the phosphate group in a configuration more amenable to an in-line attack mechanism.

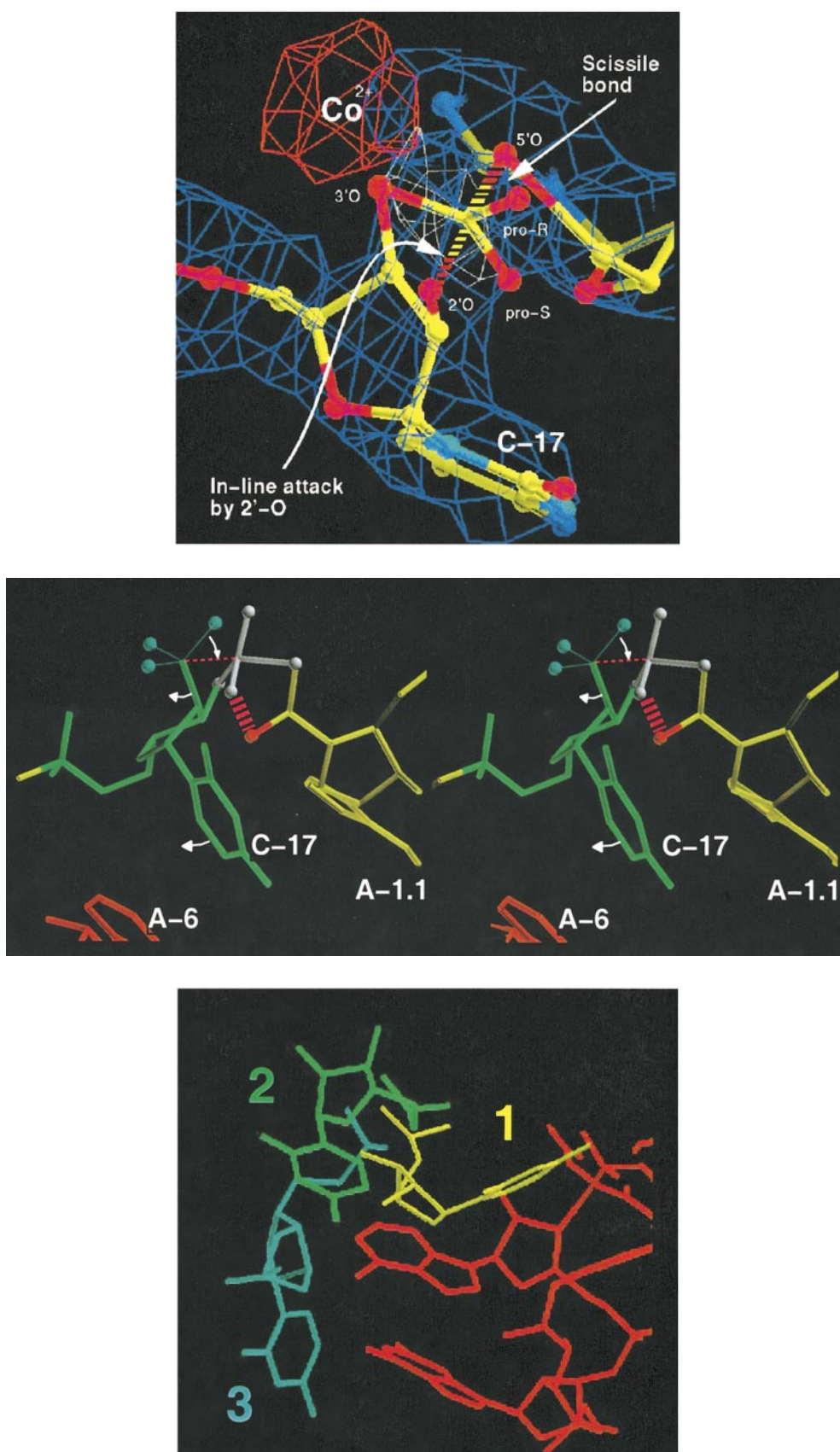


Figure 6 (legend opposite)

these reasons, we believe the conformational change required for cleavage is approximated by the structure (URX071⁹) observed using crystallographic freeze-trapping combined with a leaving-group modification (*talo*-5'-methyl-adenosine at position 1.1) that slows cleavage appreciably.⁹

Based upon the observed conformational change of C17 (Figure 5(a)), and the location of the scissile phosphate group center of mass deduced from a 3.1 Å resolution difference Fourier map (Figure 5(b)),⁹ we can propose an approximate transition-state structure (Figure 6(a)) that might form in the absence of the leaving-group modification. This proposed transition-state structure is consistent with the observed conformation and the positions of the 2' oxygen attacking nucleophile and cleavage-site phosphate atoms. Although such a structure satisfies the first requirement for an in-line attack mechanism, in terms of the positions of the relevant atoms, our analysis of the second requirement, that of orbital steering, permits us to suggest that the catalytic efficiency might be enhanced further by orienting one of the oxygen lone-pair orbitals in such a way that overlap with an empty phosphorus d-orbital would be maximized.

Unfortunately, it is impossible to visualize an active transition-state structure using X-ray crystallography due to the evanescence of its existence. The best we can hope for is a good transition-state analogue or at least a modified enzyme-substrate structure that allows observation of an accumulated conformational or chemical intermediate that normally exists only transiently. By definition, such analogues or modified structures must somehow differ from the active transition-state structure in order to have a lifetime sufficiently prolonged as to allow their observation. In the case of the hammerhead ribozyme, modification of the 5' carbon atom adjacent to the leaving-group oxygen atom in the reaction by adding an extra methyl group in the *talo*- rather than *allo*- stereoisometric configuration creates a "kinetic bottleneck" at the point of bond scission in the cleavage reaction.⁹ It is therefore likely that this modification somehow alters or prevents formation of the required transition-state structure.

Examination of the conformationally changed structure, including the atomic positions as well as the orientation of the lone-pair electrons on the attacking O2' nucleophile, indicates that the observed rigid-body pivoting motion about the β -angle of the C17 phosphate group would be required to continue along the trajectory determined by comparing the initial-state and conformational intermediate structures (Figure 5(a)). The additional small rigid-body rotation of C17, as indicated by the curved white arrows in Figure 6(b), is consistent with the rotational trajectory observed when progressing from the initial structure to the conformational intermediate and product structures (Figure 6(c)). This comparatively small change in atomic positions would then favor formation of a bond involving an electron lone pair on the 2' oxygen attacking nucleophile with the adjacent phosphorus atom at a geometrically acceptable angle. However, an additional opportunity for catalytic enhancement should result from simultaneous orienting of a 2' oxygen lone-pair orbital with respect to the axis formed by O2' and C2'. It is possible that a functional group of the RNA, or an associated cation, might perform this task. Alternatively, simply the absence of interactions that prevent nucleophilic attack, such as the two hydrogen bonds observed to orient the oxygen lone-pairs at the A9 phosphate group, may be sufficient to promote about a 1000-fold catalytic rate enhancement. Unrestricted rotation of the three sets of oxygen lone-pair orbitals about the axis containing O2' and C2' would still permit bond formation to take place, albeit somewhat more inefficiently than if the orbitals were steered favorably. In any case, the small additional rigid-body rotation of the cleavage-site nucleotide will clearly be required for optimum catalytic efficiency.

It is therefore likely that catalysis at the scissile phosphate group in the hammerhead ribozyme conformational intermediate structure is attenuated because the additional small rotation required to position the relevant atoms and then orient one of the oxygen lone-pair orbitals in-line for attack (the thin red broken line in Figure 6(b)) is prevented by the modification of the hammerhead ribozyme with the extra methyl group. This extra methyl

Figure 6. (a) The trapped conformational intermediate is roughly compatible with the future formation of an in-line trigonal bipyramidal transition state in which the attacking and leaving group oxygen atoms occupy the axial positions of the pentacoordinated oxyphosphorane. Actual formation of the transition state, however, is prevented by steric hindrance involving the added methyl group, shown in red in (b), and the *pro-S* phosphate oxygen atom when the latter tries to adopt its required equatorial position in the trigonal bipyramidal transition state. Formation of the hindered transition state would require the simultaneous continued motion of the base and ribose of C17 to position the electron lone pairs of the attacking nucleophile (the 2' oxygen atom of C17) in the orientation required for bond formation. This motion is consistent with that required to reach the observed cleavage product structure, as shown in (c). An animated gif "movie" corresponding to (c) can be viewed at <http://chemistry.ucsc.edu/%7Eewgscott/pubs/movies2.html>. Because formation of the required transition-state geometry is sterically hindered, the crystallographically observed conformational change⁹ is most likely that immediately preceding formation of the activated complex.

group, the red atom in Figure 6(b), occupies the talo-position at C5' of A1.1, and therefore sterically hinders formation of a trigonal bipyramidal transition state by clashing with (and therefore repelling) the *pro-S* non-bridging oxygen scissile phosphate group, as shown by the thick red broken line in Figure 6(b). This observation also explains why the methyl group as the talo- rather than the allo- isomer inhibits the cleavage reaction; no such clash would exist with the phosphate oxygen atoms if the modified leaving-group 5'-methylated ribose was of the allo- configuration (Figure 6(b)). Because the *pro-S* oxygen atom is repelled by an unfavorable contact with the extra methyl group, it is confined to its initial position, and therefore in turn likely repels the electron lone pair on the 2' oxygen attacking nucleophile, thus preventing optimal overlap between the lone-pair orbital on O2' and the vacant phosphorus d-orbital with which it could potentially combine. The conformational intermediate structure is thus poised for attack, pending the required small further rotation of nucleotide C17, but this further adjustment of atomic positions is prevented by a steric clash with the extra methyl group.

Prevention of catalysis by orbital steering: a complementary test of the hypothesis

Orbital steering was originally proposed as an explanation for the extraordinary catalytic power of protein enzymes,^{11,12,23} but should be equally applicable to the case of RNA enzymes. Although intuitively appealing from the perspective of a chemist, and inspired by the seminal work by Woodward & Hoffmann²³ on the conservation of orbital symmetry in pericyclic reactions, the orbital steering hypothesis has not enjoyed widespread acceptance.^{24–29} This is likely because definitive experimental evidence requires detailed structural information about the enzyme-substrate or enzyme-intermediate complex as a starting point for analysis of the hypothesis,¹³ and because it requires an accurate picture of the transition-state of the reaction being catalyzed, including the transition-state stabilizing interactions with the enzyme.³⁰ Furthermore, an accurate procedure for correlating structural interactions with catalytic enhancement must be devised. But, as mentioned in the previous section, the lifetime of a transition state prohibits its direct observation *via* X-ray crystallography, and observable intermediates or transition-state analogues must differ in some way from the true transition state, or they would not be stable enough to observe. This further complicates analyses of the contribution of orbital steering to catalysis.

One way around the difficulties for testing the orbital steering hypothesis is to examine how a reaction that occurs at a slow but significant background level, such as the base-mediated spontaneous self-cleavage of RNA, might be prevented by orbital steering. If favorable orientation of mol-

ecular orbitals is required for catalytic enhancement, it must be true that enforced unfavorable orientation of molecular orbitals must contribute to suppression of catalysis. Fortuitously, the hammerhead ribozyme prevents catalysis at a phosphate group (at A9) whose conformation appears to be quite compatible with the known requirements for atomic positioning in the in-line attack mechanism. As discussed above, correct atomic positioning apparently is a necessary but not a sufficient criterion for RNA self-cleavage, and the reactivity of the A9 phosphate group is suppressed by orbital steering insofar as the two hydrogen bonds involving the O2' of G8 steer the lone-pair orbitals into positions where they are orthogonal to the vacant d-orbitals of the phosphorus atom, despite the close proximity (2.77 Å) of the reacting atoms and the otherwise favorable angle of attack ($\sim 170^\circ$) between O2', P and O5', as shown in Figure 4.

Jencks & Page²⁹ have objected to the idea that orbital steering can provide a significant rate enhancement, suggesting instead that any such effects are subsumed under translational entropy effects and conformational effects. In the present example, however, orbital steering effects are fortuitously uncoupled from conformational effects (the A9 phosphate group has an in-line fitness parameter of $F > 1.0$), and since the reaction is unimolecular (i.e. there is only one substrate), elimination of translational entropy cannot account for the observed catalytic rate enhancement. Despite the ideal conformation of the A9 phosphate group, cleavage at this site is essentially undetectable (i.e. is slower than $\sim 10^{-7}$ per minute), although it would be predicted⁸ to be of the order of 10^{-3} per minute or greater, based upon the positions of the relevant atoms. Hence it appears, conservatively, that at least three orders of magnitude rate reduction are obtained from pure orbital steering effects alone, and therefore a similar, further enhancement in the magnitude of the rate might be obtained by removing the orientational constraints placed upon the O2' lone-pair orbitals. Furthermore, if one of the electron lone pairs was instead steered toward the scissile phosphorus atom in such a way that it would be constrained to have an orientation facilitating maximum orbital overlap, the magnitude of the rate enhancement should be significantly greater than 10^3 -fold and may be as high as 10^6 -fold, assuming a completely symmetric relationship between catalytic inhibition and catalytic enhancement. At the very least, orbital steering effects seem to be responsible for at least a 10^3 -fold rate enhancement for a phosphodiester linkage that is already optimally positioned with respect to the atoms of the phosphate group and the attacking nucleophile. Since the in-line fitness of the phosphate group, with O2' orbital steering contributions averaged out, may be responsible for a 10^4 -fold ± 10 -fold cleavage rate enhancement,⁸ it is possible that a conformational change at C17, combined with favorable orbital steering, could account for much, if not all, of the observed cataly-

tic enhancement ($\sim 10^6$ -fold) in the hammerhead ribozyme, even if the individual free energy contributions of these effects are not strictly independent and additive.

Two of the most significant criticisms of the orbital steering hypothesis can be addressed in light of our observation that orbital steering prevents RNA cleavage at the A9 phosphate group in the hammerhead ribozyme. The first of these objections is that the premises of the theory of orbital steering were derived, in part, from incorrect models.²⁸ The second is that orientation effects have been said to be of minor importance because the rather diffuse shapes of orbitals suggest that the angular dependence of orbital overlap should be rather weak.^{24,25} In the absence of a prior theory of orbital steering, the A9 phosphate example would have compelled us to conclude that cleavage could be prevented only by orientation of the 2' oxygen atom in such a way as to prevent orbital overlap between the lone electron pairs and the d-orbitals of the phosphorus atom. In other words, we would have concluded that the hydrogen bonding pattern was responsible for catalytic suppression *via* an orbital orientation mechanism, and would have thus postulated that such an effect could be employed to enhance catalysis through favorable orbital orientation that maximized overlap. Because the orientations of the lone-pair orbitals are fixed by the hydrogen bonding scheme at 90° and 120° away from the phosphorus atom, the orbital angular dependence of reactivity is maximized in much the same way as the solvent cage can maximize this effect, as reported.²⁶ Based on the latter observation, it is likely that orbital steering, either within the confines of a solvent cage, or perhaps within the "pre-arranged solvent cage" of an enzyme active site (consisting of hydrogen bonds between the enzyme and the substrate and/or activated complex), can significantly enhance catalysis.

Orbital steering effects in the context of complementary catalytic strategies

Although we have argued that the magnitude of the orbital steering effect alone might account for a large fraction of the observed catalytic rate enhancement in the hammerhead ribozyme relative to uncatalyzed RNA self cleavage, it is likely that other, well-recognized catalytic strategies are employed by the hammerhead ribozyme to accelerate RNA self-cleavage. These other effects include, for example, general acid/base catalysis and transition-state stabilization interactions.

The reaction catalyzed by the hammerhead ribozyme is the same as the first step of the RNA cleavage reaction catalyzed by RNase A, one of the best-characterized protein enzymes. The canonical roles of three active-site amino acid residues, His12, His119 and Lys41, are those of a general base catalyst, a general acid catalyst, and a catalytic interaction that stabilizes the excess negative charge that accumulates in the oxyphosphorane

transition state.³¹ However, some debate exists over the microscopic mechanism by which acid/base catalysis and transition-state stabilization takes place.^{32,33} In any case, the analogy with RNase A leads quite naturally to the proposal that hydrated divalent metal ion complexes, like $[\text{Mg}(\text{H}_2\text{O})_6]^{2+}$ and $[\text{Mg}(\text{H}_2\text{O})_5(\text{OH})]^+$, play the roles of general acid and general base catalysts, respectively,^{34,35} when specifically bound to the RNA. In addition, the same or another divalent metal ion can be invoked to catalyze transition-state stabilization by binding *via* an inner-sphere interaction (i.e. as a Lewis acid) to one of the non-bridging phosphate oxygen atoms of the scissile phosphate group before or during catalysis in order to balance the excess negative charge that accumulates in the oxyphosphorane transition state.³⁴

Our recent finding that the hammerhead ribozyme, as well as the hairpin ribozyme and the VS ribozyme do not require divalent metal ions for efficient catalysis,³⁶ permits us to suggest that in these three ribozymes (at least), the RNA itself must supply the functional groups required for acid/base catalysis and transition-state stabilization if these types of catalysis are required. Although we were not able to observe efficient self-cleavage of the HDV ribozyme in the absence of divalent cations, it has since been shown that C75, rather than a divalent cation, plays the role of a general acid catalyst.³⁷⁻³⁹ The X-ray crystal structure of the 50 S subunit of the ribosome^{40,41} verified the prediction⁴² that the ribosome is a ribozyme, and A2451 has been implicated as an acid/base catalyst in the peptidyl transfer reaction.^{40,43} No divalent cation appears to be close to the peptidyl transferase active site. The recent crystal structure of the hairpin ribozyme⁴⁴ reveals an active-site guanosine base that makes two hydrogen bond contacts to the cleavage site on the substrate. The N1 atom of G8 donates a hydrogen bond to the 2' oxygen-attacking nucleophile, and the exocyclic amine group donates a hydrogen bond to one of the non-bridging scissile phosphate oxygen atoms. Thus G8 may play a role as both an acid/base catalyst (the $\text{p}K_a$ of the N1 proton is about 9.5) and a transition-state stabilization catalyst. The enzyme-product structure of the hammerhead ribozyme¹⁰ revealed potential interactions between the exocyclic amine group of A6 and a non-bridging scissile phosphate oxygen atom, as well as G5 and the 2' oxygen atom that may be relevant to transition-state stabilization and acid/base catalysis, respectively.

Acid/base catalytic interactions and transition-state stabilization reactions, however, may not be completely independent of orbital steering interactions. For example, the hairpin ribozyme crystal structure⁴⁴ shows two hydrogen bonds between G8 and the cleavage site (Figure 7). In addition to the potential catalytic roles already described, these hydrogen bonds appear to steer the 2' oxygen lone pairs as well as to fix the atomic positions

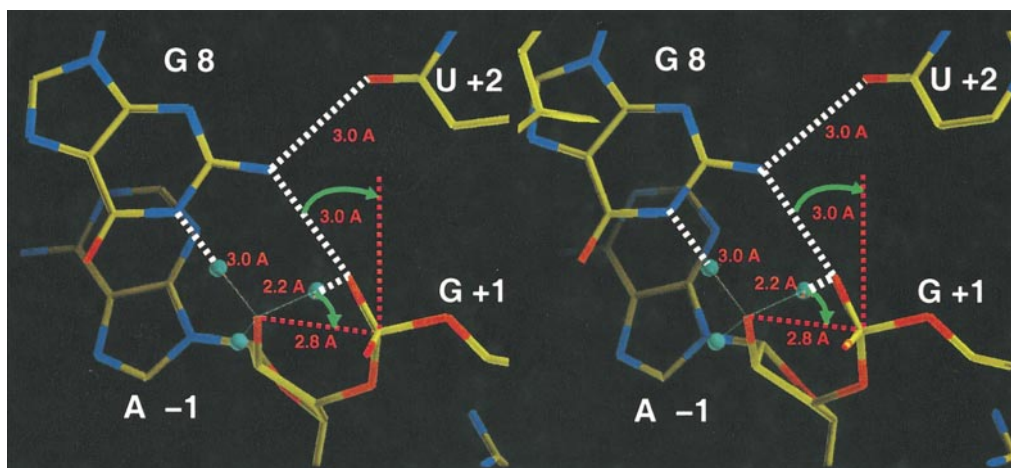


Figure 7. Stereoview of the hairpin ribozyme active site, showing hydrogen bonding interactions between G8 and the cleavage-site phosphodiester linkage. The vertical red broken line indicates the phosphate oxygen atom position in a trigonal bipyramidal transition state, and the nearly horizontal red broken line indicates the direction of in-line attack of the 2' oxygen atom upon the scissile phosphorus atom. Movement of the G8 U +2 base-pair to the right in such a way that the hydrogen bonds line up with the red broken lines will facilitate in-line attack by directing the oxygen atom lone-pair electrons toward the phosphorus atom as well as formation of the pentacoordinated trigonal bipyramidal transition state. G8 may also function as a general base catalyst.

of one of the scissile phosphate non-bridging oxygen atoms and the 2' oxygen atom. As cleavage is initiated, it is required that the non-bridging phosphate oxygen atoms move into the axial plane, in turn requiring a small movement of G8 if the hydrogen bond is to be maintained. If both hydrogen bonds are maintained, the small movement of G8 relative to the 2' oxygen position will steer one of the other lone-pair orbitals directly toward the scissile phosphorus atom, thus optimizing the orbital orientation and overlap, facilitating bond formation. Clearly, the two effects might be highly coupled, and although physically distinct, would be inextricably linked to the same nucleotide movement.

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