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Biophysical and biochemical investigations of RNA catalysis in the hammerhead ribozyme

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1. How do ribozymes work?

The discovery that RNA can be an enzyme (Guerrier-Takada *et al*. 1983; Zaug & Cech, 1986) has created the fundamental question of how RNA enzymes work. Before this discovery, it was generally assumed that proteins were the only biopolymers that had sufficient complexity

and chemical heterogeneity to catalyze biochemical reactions. Clearly, RNA can adopt sufficiently complex tertiary structures that make catalysis possible. How does the three-dimensional structure of an RNA endow it with catalytic activity? What structural and functional principles are unique to RNA enzymes (or ribozymes), and what principles are so fundamental that they are shared with protein enzymes?

2. The hammerhead RNA as a prototype ribozyme

The hammerhead ribozyme in many respects is the 'serine protease of RNA enzymes' in that it is a comparatively simple and well-studied ribozyme that in principle should be capable of revealing the secrets of its catalytic potential if we are able to pose the right questions and carry out useful and informative experiments. Much attention has been focused upon this particular ribozyme with the hope that if its catalytic properties become well-understood, our grasp of the phenomenon of RNA catalysis in general will become more comprehensive so that generalizations may appear that are applicable to the larger ribozymes, to RNA splicing and peptidyl transfer, and perhaps even beyond to a unified understanding of RNA and protein enzymology.

2.1 RNA enzymes

RNA catalysis was originally discovered in Group I intron pre-ribosomal RNA catalyst (Kruger *et al*. 1982; Zaug & Cech, 1986) and in the RNA subunit of RNase P (Guerrier-Takada *et al*. 1983). This discovery impels us to answer the question of how RNA catalysis works. The belief that all enzymes have to be composed of proteins crumbled in the early 1980s with the discovery that RNA can, by itself, catalyze fairly complex splicing reactions (*via* the Group I and Group II introns) and tRNA processing reactions (*via* RNase P, an RNA–protein complex whose RNA subunit is enzymatically active). Thus the problem of how RNA, with but four relatively inert bases, can function as a biological catalyst has become a fundamental question of molecular biology.

By understanding how ribozymes work, we may also learn more about how life originated. RNA may have been the original self-replicating pre-biotic molecule, according to the 'RNA World' hypothesis (Gesteland & Atkins, 1993), potentially catalyzing it own replication. Understanding the fundamental principles of ribozyme catalysis therefore may also give us new insights into the origin of life itself. The answer to the question of how ribozymes work also has practical consequences. as RNA enzymes are particularly well-suited for design as targeted therapeutics for a variety of diseases (for a recent review, see Vaish *et al*. 1998).

2.2 Satellite self-cleaving RNAs

In addition to the Group I and Group II introns and RNase P, several smaller catalytic RNAs have since been discovered, including the hammerhead (Prody *et al*. 1986), hairpin (Hampel & Tritz, 1989), *Neurospora* VS (Guo *et al*. 1993) and hepatitis delta virus (Sharmeen *et al*. 1988) self-cleaving motifs. These four self-cleaving RNAs are all involved in virusoid or satellite virus RNA replication. Although these self-cleaving RNAs have very different sequences and structures, all catalyze the same chemical reaction, phosphodiester bond scission. Virusoid and satellite RNAs are small circular, single-stranded RNAs that are virus-like entities (reviewed in Symons, 1992) found in association with several types of plant RNA virus (such

as tobacco ringspot virus) and, in the case of the hepatitis delta virus (HDV), in association with hepatitis B. These small circular RNAs rely upon the cellular machinery of the host as well as products of viral infection to replicate via a rolling-circle mechanism. The covalently closed single strand of RNA is a template for an RNA polymerase that creates a complementary copy of the circular molecule. However, this molecule will be linear and, as the polymerase travels along the RNA for several revolutions, a long linear concatameric complementary copy of the circular template is produced. To complete the replication cycle, the linear concatamer must be separated into linear momomers, and these monomeric complementary copies of the original circular RNA must then close up to form circular molecules. These can then undergo the same sort of rolling-circle replication, with concomitant production of linear concatameric copies of the original circular template. These again must be divided into linear monomeric fragments which again will circularize and ligate to form covalently closed circular copies of the original satellite RNA. The linear concatamers are cleaved into monomeric fragments autolytically, i.e. without the intervention of any enzymes or other intermolecular species, with the possible exception of divalent cations. (Recently, a protein has been identified that may aid in this process by binding to the RNA (Luzi *et al*. 1997), but its presence is not essential for the self-cleavage reaction to take place *in vitro*.)

A relatively small, autonomously folding motif of RNA found at the cleavage-site junction is responsible for catalyzing a highly sequence-specific self-cleavage event in each case. In the case of the satellite RNA of tobacco ringspot virus, for example, an approximately 60 nucleotide sequence that has been dubbed the 'hairpin' self-cleaving RNA is found at the junction of two monomeric sequences in the linear concatameric complementary copies of the original circular satellite RNA, and a different sequence of approximately 50 nucleotides, called the 'hammerhead' self-cleaving RNA, is found at the analogous positions in the concatameric copy of the original sequence produced in the second phase of the rolling-circle replication. These self-cleaving motifs reappear in a variety of other satellite RNA species. Similarly, HDV is a single-stranded satellite RNA virus associated with hepatitis B, and the HDV self-cleaving RNA, again consisting of an autonomously folded region of about 80 nucleotides, is involved in the rolling-circle replication of the hepatitis delta virus. Finally, the VS self-cleaving RNA is a motif of about 160 nucleotides involved in the rolling-circle replication of a retroplasmid in *Neurospora*. In each case, the self-cleaving RNA catalyzes a highly sequence-specific phosphodiester bond cleavage reaction that yields monomeric fragments having 5′-hydroxyl and 2′,3′-cyclic phosphate termini. Each monomeric fragment can then recircularize when the two ends of the monomer approach one another and the complete folding motif is regenerated. The ends are ligated when the self-cleaving RNA catalyzes the reverse chemical reaction, that is, ligation of the phosphodiester backbone. hence the RNA is catalytic in the sense that cleavage is highly specific, greatly accelerated over the background rate of the reaction, and is reversible. However, these are not a true enzymatic catalysts in the technical sense because the catalyst are not regenerated in such a way that true multiple turnover in the presence of an excess of substrate occurs. The natural biological reaction is a single-turnover cleavage event and a single-turnover ligation event.

The hammerhead, hairpin, VS and HDV self-cleaving RNAs can be made into true RNA enzymes, however, by a trivial alteration of their phosphodiester bond connectivities in such a way that a single-strand of RNA corresponding to the autonomous folding motif is divided into two strands, one of which (the substrate strand) gets cleaved by the other. When this is

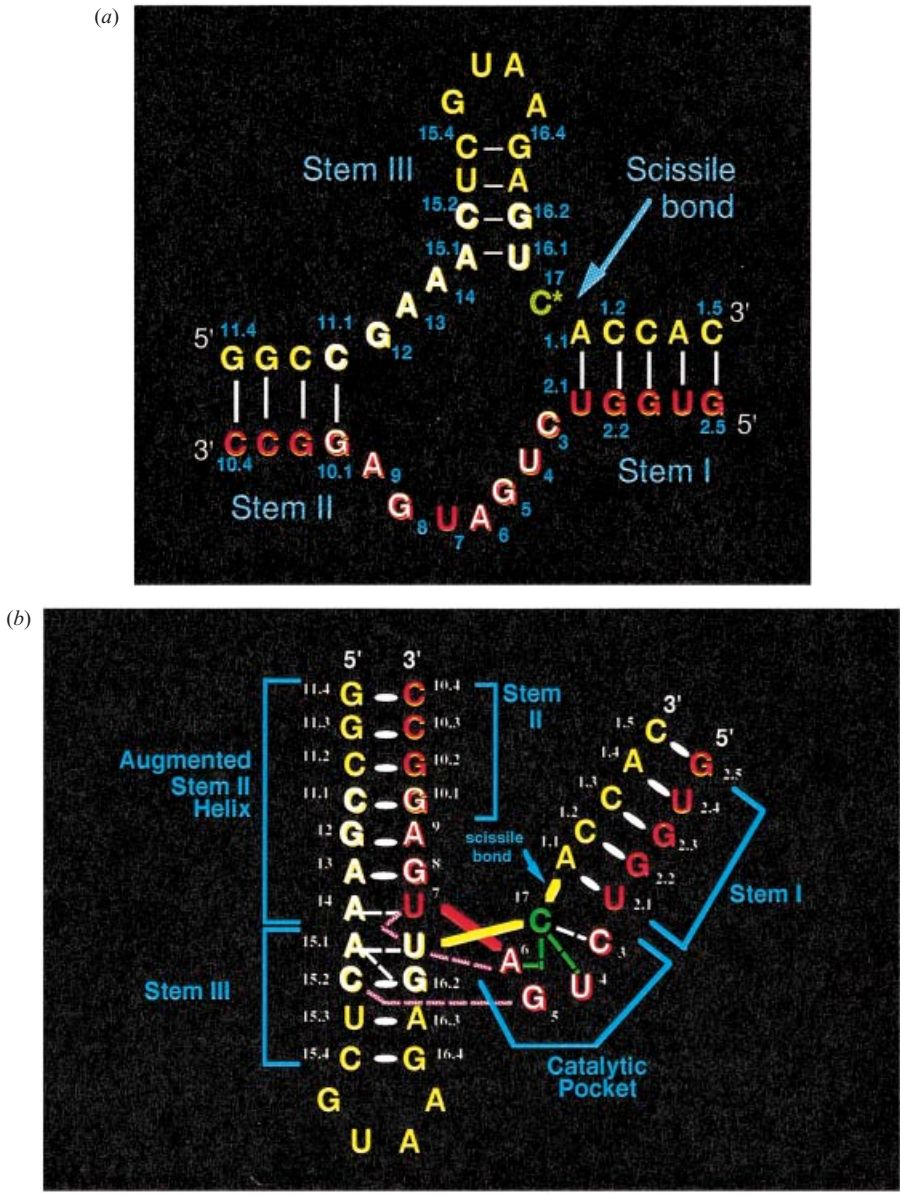


Fig. 1. For legend see facing page.

done, these four small self-cleaving RNAs become true ribozymes that catalyze multiple-turnover cleavage reactions with the kinetic properties typically observed with true protein enzymes.

2.3 Hammerhead RNAs and hammerhead ribozymes

Hammerhead RNAs are small self-cleaving RNAs that have in common a conserved motif found in several of the viroids and satellite RNAs that replicate via a rolling circle mechanism as described above. The hammerhead motif consists of three base-paired stems flanking a central core of 15 conserved nucleotides, as depicted in Fig. 1 (Uhlenbeck, 1987; Ruffner *et*

(*c*)

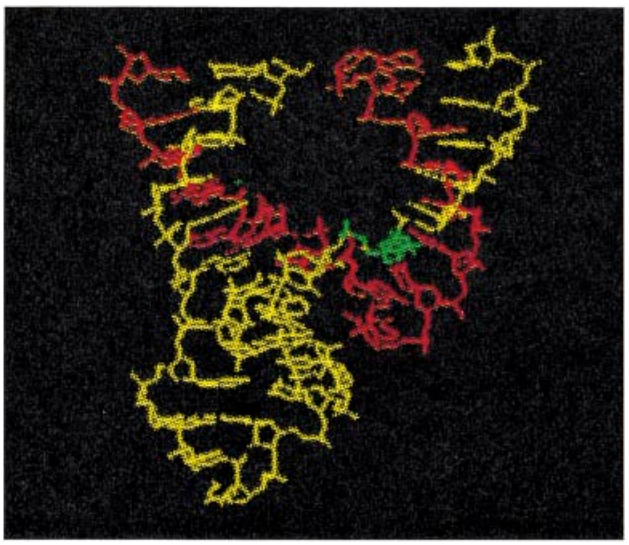


Fig. 1. (*a*) Canonical secondary structure of the hammerhead ribozyme in the I/II format, showing the numbering convention (Hertel *et al*. 1992). This particular sequence was optimized for crystallization rather than catalysis. The conserved nucleotides are shown as double letters. The enzyme strand is in red, and the substrate strand is in yellow, with the cleavage site nucleotide highlighted in green. The scissile phosphate is the one 3′ to the cleavage site nucleotide; i.e. it is the phosphate on residue 1·1 in Stem I. The helices are also named according to convention. (*b*) Secondary structure of the hammerhead ribozyme in the I/II format that reflects the arrangement found in the crystal structures. Note the arrangement of Stems I, II and III and the additional pairings between several of the conserved bases of the core region that are shown as single-stranded regions in (*a*). (*c*) The corresponding three-dimensional crystal structure of the hammerhead ribozyme.

al. 1990; Symons, 1992). The conserved central bases, with few exceptions, are essential for ribozyme's catalytic activity.

Naturally occurring hammerhead RNAs are a single covalent macromolecule before self-cleavage, with the core region connected by stem-loop structures on two of the three helices, and the remaining helix joins with the remainder of the RNA molecule. If either the Stem II or Stem III connecting loop is retained while the loop connecting the other two helices is eliminated artificially, the resulting RNA molecule is composed of two separate covalent strands of RNA, one of which gets cleaved. This two-stranded system is a true ribozyme in that the cleavable strand (the substrate strand), when supplied in excess, will be cleaved by the other strand (the enzyme strand) in a multiple-turnover process that obeys Michaelis–Menton kinetics. Hammerhead ribozymes in which Stem II is connected by a loop are called 'format I/III' ribozymes and those in with a loop on Stem III are called 'format I/II' ribozymes. The first hammerhead ribozyme constructed in this way was a format I/II ribozyme (Uhlenbeck, 1987), but the format I/III ribozyme (Haseloff & Gerlach, 1988) is perhaps a more intuitively appealing division in that only two of the conserved nucleotides in this case appear in the substrate strand. Both exhibit standard enzyme kinetics behavior, but the format I/III ribozymes often tend to be more kinetically well-behaved in the sense that the substrate is often less prone to forming alternative inhibitory structures.

Although division of the hammerhead RNA into enzyme and substrate strands for the convenience of experimental investigators gives rise to a catalytic system that conforms to the

usual kinetic properties of protein enzymes, it should always be kept in mind that the true hammerhead RNA motif is a single-turnover self-cleaving molecule that cleaves only upon folding of both components, whether or not they are covalently connected by a distant loop. This situation differs from that of the typical protein enzyme, in which the enzyme is pre-folded before binding the substrate, and comparatively minor structural rearrangements usually take place (induced fit) upon substrate binding. The enzyme strand of the hammerhead ribozyme will not be pre-folded, but rather the enzyme and substrate must fold together in an interdependent manner to assemble the catalytic core, and only then may catalysis take place. In that sense it is essentially accidental that the hammerhead ribozyme obeys Michaelis–Menton kinetics, and if the analogy with pre-folded protein enzymes is always assumed, some tenuous conclusions about the differences between protein and RNA catalysis might be inferred. For example, it has been reported recently that the unusual sensitivity of the hammerhead ribozyme to mutations that disrupt the base stacking interactions imply that the hammerhead ribozyme is similar in its properties to a denatured protein (Peracchi *et al*. 1996, 1998). This observation appears to be unexpected in the context of protein enzymology but may simply be a restatement of the fact that the enzyme strand of the hammerhead RNA is dependent upon the substrate RNA to fold and associate with it to form the catalytic core. Hence the artificial division between enzyme and substrate in the small ribozymes must always be kept in mind.

The hammerhead ribozyme is arguably the best-characterized ribozyme. Its small size, thoroughly investigated cleavage chemistry, known crystal structure, and its biological relevance make the hammerhead ribozyme particularly well-suited for biochemical and biophysical investigations into the fundamental nature of RNA catalysis. Despite the extensive structural and biochemical characterization of the hammerhead ribozyme, many important questions remain about how this RNA molecule's structure enables it to have catalytic activity. Our understanding of the relationship between the structure of the hammerhead RNA and its catalytic activity therefore remains rather conjectural. The hammerhead is currently the only ribozyme whose catalytic activity has been characterized in terms of structural changes that take place in the crystal upon initiation of the self-cleavage reaction (Scott *et al*. 1996; Murray *et al*. 1998a), and it therefore offers the best hope of understanding how RNA structure activates catalysis.

3. The chemical mechanism of hammerhead RNA self-cleavage

The hammerhead ribozyme self-cleavage reaction is deceptively simple. Like the nonezymatic alkaline cleavage of RNA that is responsible for its inherent instability, the hammerhead RNA self-cleavage reaction is simply a phosphodiester isomerization from a 5′ to 3′ diester to a 2′,3′-cyclic phosphate diester, resulting in the cleavage of the phosphate backbone. Since a water molecule is not added at the point of cleavage, the reaction is even more simple than a hydrolysis reaction. By preserving the phosphodiester character of the cleavage-site phosphate, the hammerhead RNA ensures that the self-cleavage reaction is thermodynamically reversible, a condition that is critical for single-stranded rolling circle nucleic acid replication as noted above. Despite the fact that this reaction is perhaps the simplest chemical transformation that an RNA molecule may undergo, and is in many

respects the same as the random uncatalyzed alkaline cleavage reaction that is responsible for slowly degrading RNA, it has two important differences. First, the sequence specificity of the catalyzed reaction is absolute, and second, the rate of the reaction is significantly enhanced over what one would expect for the random degradation of RNA. Nonetheless, heated debate over the details concerning the hammerhead ribozyme mechanism and the interpretation of experimental results compel us to consider the details carefully, with the hope of obtaining a satisfactory understanding of this simple prototypical ribozyme reaction.

3.1 Phosphodiester isomerization via an $S_N2(P)$ reaction

RNA spontaneously degrades very slowly even in the absence of divalent metal ions and enzymes (protein or RNA) that catalyze cleavage of the RNA. This spontaneous process, termed alkaline cleavage, accelerates as pH is elevated, suggesting that deprotonation of the 2′-OH initiates the cleavage reaction. It is essentially nonspecific with respect to the RNA sequence, but occurs to a greater extent in nominally unstructured or more flexible regions of RNA than in A-form helices. In other words, the helical conformation of RNA serves to protect it from spontaneous random alkaline cleavage. The alkaline cleavage reaction proceeds by an 'in-line' or $S_N2(P)$ reaction in which the attacking nucleophile (the 2′-oxygen) must be in line with the phosphorus atom of the adjacent phosphate as well as with the 5′-oxygen of this phosphate (the leaving group in the displacement reaction). This arrangement ensures that in the trigonal bipyramidal transition-state structure (a pentacoordinated oxyphosphorane) that is then formed, the attacking and leaving group oxygens will both occupy the two axial positions, as is required for an S_N2 reaction mechanism. The phosphates of a helical nucleic acid are, however, in a conformation (antiperiplanar gauche) that is incompatible with this mechanism; the 2′-oxygen and 5′-oxygen atoms will make a 90° angle with the phosphorus (or are 'adjacent') in a pentacoordinated trigonal bipyramidal transition-state structure in which the 5′-oxygen leaving group occupies an equatorial position. Attack of the 2′-oxygen upon the nearest phosphorus would therefore require production of an oxyphosphorane intermediate of sufficiently long lifetime to allow a 'pseudorotation' to bring both the attacking and leaving group oxygens to the axial positions. Such a reaction would proceed with retention of configuration about the phosphorus (Westheimer, 1968), whereas a simple $S_N2(P)$ mechanism entails that the reaction proceeds with inversion of configuration. The nonezymatic cleavage of RNA is in fact observed to proceed with inversion of configuration, as does the hammerhead-catalyzed cleavage reaction. Hence RNA that adopts the helical conformation, in so doing, protects the phosphodiester backbone from alkaline cleavage relative to random-coil RNA in which conformations allowing in-line attack to occur are more accessible via structural fluctuations (Soukup & Breaker, 1999).

As with the nonezymatic cleavage of RNA, the hammerhead cleavage reaction proceeds via an in-line or $S_N2(P)$ mechanism in which the attacking 2′-oxygen displaces the 5′-oxygen at the cleavage phosphate. The cleavage products also have 2′,3′-cyclic phosphate and 5′-hydroxyl termini (Buzayan *et al.* 1986; Hutchins *et al.* 1986), as in the case of nonezymatic alkaline cleavage of RNA. Unlike the case of nonezymatic RNA cleavage, the hammerhead ribozyme catalyzes a highly sequence-specific cleavage reaction with a typical turnover rate of approximately 1 molecule of substrate per molecule of enzyme per minute at pH 7·5 in 10 mM Mg^{2+} (so-called 'standard reaction conditions'), depending upon the sequence of the particular hammerhead ribozyme construct measured. This represents an approximately

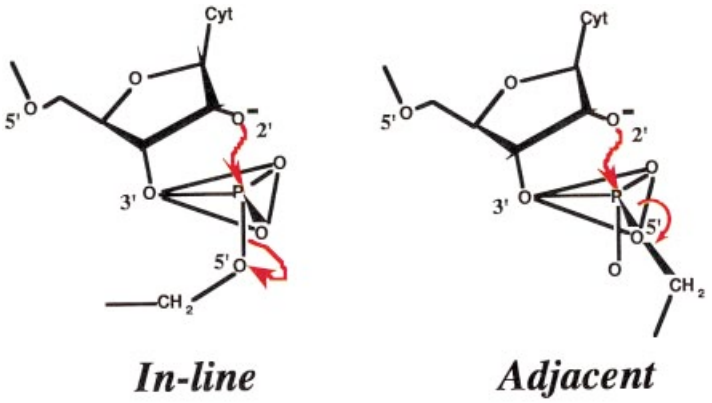


Fig. 2. The different phosphate backbone conformations required for an 'in-line' (or S_N2) vs. an 'adjacent' cleavage mechanism.

10000-fold rate enhancement over the nonezymatic cleavage of RNA. The $S_N2(P)$ mechanism of cleavage in the hammerhead ribozyme has been demonstrated by three independent laboratories who have shown that the reaction proceeds with an inversion of configuration of the nonbridging phosphate oxygen atoms about the scissile phosphorus atom (van Tol *et al*. 1990; Slim & Gait, 1991; Koizumi & Ohtsuka, 1991). In each case, inversion of configuration was demonstrated using thio-substituted non-bridging phosphate oxygens. In the cases of at least 10 protein enzymes, inversion or retention of configuration has been demonstrated using thio-substituted nonbridging phosphate oxygens and confirmed using isotopically labeled nonbridging oxygen atoms without disagreement between the two approaches. (Eckstein, 1985). By analogy, it is therefore most likely that an $S_N2(P)$ mechanism also pertains to unmodified hammerhead ribozymes, and is not simply an artifact of phosphorothioate substitution, as such artifacts have never been observed previously.

The cleavage-site phosphate (and several others) in the hammerhead ribozyme also shows a significant thio-effect. Substitution of the *pro*-R phosphate oxygens with sulfur at the scissile phosphate, the G-8, A-9, A-13 and A-14 phosphates all interfere significantly with hammerhead ribozyme catalysis (Ruffner *et al*. 1990), whereas substitution of the *pro*-S phosphate oxygen of the scissile phosphate has a much less profound effect (Slim & Gait, 1991; Zhou *et al*. 1996a). The nonenzymatic alkaline cleavage of RNA, by contrast, shows no significant thio-effects (Burgers & Eckstein, 1979; Herschlag *et al*. 1991), indicating that the catalyzed self-cleavage reaction must in some way be mechanistically distinct from the noncatalyzed reaction. Moreover, the scissile and A-9 phosphates both show a 'rescue' effect in which more thiophilic metal ions such as Mn^{2+} and Cd^{2+} restore, or even enhance, catalysis (Ruffner *et al*. 1990; Dahm & Uhlenbeck, 1991; Zhou *et al*. 1996a; Scott & Uhlenbeck, 1999; Peracchi *et al*. 1997; Wang *et al*. 1999). The interpretation of these phosphorothioate rescue experiments is discussed in the next section.

Since the preferred conformation of RNA is an A-form helix (or a helix having noncanonical base-pairing that approximates an A-form helical geometry in many cases), it is fair to ask whether hammerhead ribozyme catalysis is achieved merely by repositioning the scissile phosphate for in-line attack from the adjacent 2′-oxygen nucleophile. Two lines of reasoning suggest that such a conformational alteration is a necessary but not sufficient

criterion for hammerhead ribozyme catalysis. First, if the hammerhead RNA folds in a manner that simply favors positioning of the ribose ring of the attacking nucleophile and the scissile phosphate in a conformation amenable to in-line attack (say, for example, by causing the cleavage-site nucleotide to be 'flipped out' of the helix), one might expect the reaction to be in every way identical to nonezymatic alkaline cleavage, apart from the observed sequence specificity and reaction rate increase. Again, the hammerhead-catalyzed cleavage reaction, unlike the nonenzymatic reaction, shows a significant thio-effect at the scissile phosphate, indicating that some other factors must be at work that make the catalyzed reaction in some way chemically distinct. Second, the crystal structure (see Section 5 below) reveals that the A-9 phosphate and the ribose of G-8 are positioned perfectly for an in-line attack of the 2′-oxygen of G-8, yet no residual cleavage has been observed at this phosphate. Clearly, the conformation of the scissile phosphate *per se* cannot be the only factor involved in hammerhead ribozyme catalytic enhancement of the RNA self-cleavage reaction. Other factors, in addition to having the phosphate in the correct conformation (i.e. what could be called the *structural* basis for catalysis), must be responsible for the *chemical* basis of hammerhead ribozyme catalysis. (The structural and chemical bases for catalysis are, of course, highly interdependent.)

The rate of cleavage for hammerhead ribozymes in constructs in which the chemical step appears to be rate-limiting is log-linearly proportional to the pH of the reaction mixture with a proportionality constant of approximately 1·0 within a pH range between 6·0 and 8·0 (Dahm *et al*. 1993). (Above pH 8·0, the rate begins to plateau.) This observation permits the suggestion that a single proton abstraction is involved in the rate-limiting step of the reaction, consistent with abstraction of the 2′-proton being rate-limiting. At this point it is appropriate to ask whether the hammerhead ribozyme reaction is sequential or concerted. A strong case based primarily upon circumstantial evidence can be made for a required conformational change within the enzyme–substrate complex prior to the chemical step(s) of the cleavage reaction (see below). However, it is not clear whether the actual cleavage reaction is itself concerted or sequential. Although an 'adjacent' mechanism would require a chemical intermediate (the pentacoordinated oxyphosphorane) to be sufficiently long-lived to support pseudorotation, as described above, and would therefore by necessity dictate that the reaction be sequential rather than concerted, the 'in-line' or $S_N2(P)$ mechanism places no such requirement upon the cleavage chemistry. In principle, the pentacoordinated oxyphosphorane may be simply a transition state (as with an $S_N2(C)$ reaction as observed in carbon chemistry) or may be a true chemical intermediate. If the $S_N2(P)$ reaction is concerted, this necessitates that the bond between the 2′-oxygen and the scissile phosphorus atom forms as the bond between the phosphorus and the 5′-oxygen simultaneously breaks, and a single pentacoordinated transition-state exists for the reaction. If the reaction is sequential, the bond between the 2′-oxygen and the phosphorus forms prior to the dissociation of the 5′-oxygen. If that is the case, then the pentacoordinated oxyphosphorane must exist as a chemical intermediate that has a finite lifetime, and it will be flanked by two transition states on the reaction coordinate. To be a true intermediate, the structure must be stable enough to have at least one bound vibrational mode, and therefore must have a lifetime that is significantly longer than the period corresponding to the frequency of this vibration. In principle, it may be possible therefore to detect a spectroscopic signature of the intermediate if it exists, or to detect it using rapid kinetics techniques, or even to trap it under favorable circumstances. To date, no such spectroscopic evidence exists for such an intermediate, and it has never been

physically isolated, but *ab initio* molecular orbital calculations indicate that such an intermediate might in fact exist (Zhou & Taira, 1998), and some evidence from hammerhead enzyme kinetics also indicates that this might be the case.

If we assume for the sake of argument that there are two (chemically reactive) transition states (TS1, corresponding to bond-formation, and TS2, corresponding to bond scission) in the nonenzymatic alkaline cleavage of RNA, one of these must correspond to the rate-limiting step. If TS1 is a higher-energy barrier, then formation of the 2′-oxygen to phosphorus bond will be rate-limiting, and if TS2 is a higher-energy barrier, then cleavage of the 5′-oxygen to phosphorus bond will be rate-limiting. According to the same molecular orbital calculations, TS2 is higher in energy than is TS1, predicting that bond cleavage is rate-limiting (Zhou & Taira, 1998). RNA in which a phosphate nonbridging 5′-oxygen is replaced with a 5′-sulfur cleaves approximately 10^5 times more rapidly than the corresponding unmodified RNA having the same sequence (Kuimelis & McLaughlin, 1995; Zhou *et al*. 1996b). Because this phosphorothioate substitution changes the leaving group character (i.e., it lowers the pK_a of the leaving group by 5 units) but not that of the attacking nucleophile, one would not expect such a profound rate enhancement if the first step of the reaction were rate-limiting. Instead, one might expect little if any rate change, since TS1 would be the kinetic bottleneck in the reaction pathway.

When the 5′-sulfur modification is incorporated into hammerhead ribozyme substrates in such a way that the leaving group of the hammerhead RNA self-cleavage reaction is thus modified, the modified substrate RNA is cleaved approximately 100 times more rapidly by the hammerhead ribozyme than is the unmodified substrate RNA. Although the differences in this case are not nearly so pronounced, the same argument in favor of the bond-breaking step being rate-limiting applies for a nonconcerted hammerhead ribozyme-catalyzed RNA cleavage reaction.

The above analysis assumes both RNA cleavage reactions are sequential. If in fact the bond-forming and bond-breaking steps occur simultaneously in a concerted reaction, the effects of the substitution of sulfur for the 5′-oxygen leaving group atom cannot be regarded as a perturbation only on the bond-breaking part of the reaction. If these two 'steps' occur simultaneously in a concerted reaction, or even if they occur sequentially in a nonconcerted reaction in which the local energy minimum corresponding to the chemical intermediate is very shallow, leaving group effects will not be neatly separable from bond formation, but will instead tend to be correlated at least somewhat, since the potential energy surface is a continuum rather than a collection of isolated, discrete energy states (Cannon *et al*. 1996). In addition, the positive log-linear dependence of reaction rate upon pH, as noted above, has been cited as evidence for proton abstraction (presumably at the 2′-OH of the cleavage site base either prior to or during bond formation) being rate-limiting. How is this to be reconciled?

Returning to the example of RNase A, the first step of the reaction (i.e., that which is analogous to the entire hammerhead reaction) is believed to be a concerted reaction, where histidines serve as both general acid and general base catalysts. The pK_a of histidine is approximately 7, and a graph of the log of the reaction rate vs. pH is a bell-shaped curve having a maximum at about pH 7. This reflects the fact that acidic conditions favor leaving-group stabilization by a doubly protonated histidine, but disfavor proton abstraction by a singly protonated histidine, and that basic conditions have the opposite effect. When the pH matches the pK_a of the histidine, the best compromise is reached and the reaction is catalyzed

in the most efficient manner. No such bell-shaped curve exists for the hammerhead ribozyme reaction, but as noted, the cleavage rate begins to plateau above pH 8 or so. If the analogy with RNase A is valid, it is tempting to suggest that the hammerhead ribozyme log rate vs. pH curve would also be bell-shaped with a maximum at a pH above say 8·5 or 9. Much above a pH of 8·5 or so, structural perturbations to RNA become significant, due to deprotonation of base functional groups, beginning with uracil. Hence the potential existence of an RNase A-like bell curve becomes problematic to test. If it is valid to infer its existence, however, several considerations may follow. First, it would suggest that the reaction is concerted, or nearly so, since both the protonation and deprotonation events would have an effect upon the reaction rate. Second, if the reaction is concerted, it resolves the paradox of how bond breaking can be rate-limiting if the rate increases as a function of pH over the range pH 6–8. Third, it suggests that the acidic and basic catalytic moieties have pK_a values that are around 9·0 or greater, implying that the catalytic species is (a) water, in its ionized form, (b) metal-bound water, or hydroxide, or (c) functional groups contributed by the RNA itself.

3.2 The canonical role of divalent metal ions in the hammerhead ribozyme reaction

The hammerhead RNA, and all other naturally occurring ribozymes, were originally believed to be obligate metalloenzymes (Dahm & Uhlenbeck, 1991; Pan *et al*. 1993; Pyle, 1993) in that they appeared to require a divalent metal ion, such as Mg^{2+}, to mediate catalytic cleavage of the RNA phosphodiester backbone. In principle, there are several opportunities for a divalent metal ion to enhance catalysis, including initiation of the reaction by base catalysis, stabilization of the transition state through interaction with a nonbridging phosphate oxygen, and enhancement of the leaving group stability through stabilization of an accumulating negative charge on the 5′-oxygen as the phosphodiester bond is cleaved. This is based upon an analogy with RNase A, an enzyme that catalyzes an RNA cleavage reaction in which the first step is chemically identical to that catalyzed by the hammerhead ribozyme. Two histidines and a lysine contribute to the active-site structure of RNase A; one histidine is doubly protonated and the other is not. The singly protonated histidine is believed to serve as a general base catalyst that abstracts the 2′-hydroxyl proton, and the doubly protonated histidine is believed to serve as a general acid catalyst that donates a proton to the 5′-oxygen as the phosphodiester bond is broken. In addition, the positively charged lysine is believed to make a direct contact with one of the nonbridging oxygens of the scissile phosphate, providing additional stabilization by helping to disperse the excess negative charge that accumulates in the transition state of the reaction.

In the case of base catalysis in the hammerhead ribozyme, a divalent metal ion is thought to serve the role analogous to the unprotonated histidine when it binds to the RNA and induces ionization of the 2′-hydroxyl at the cleavage site. The catalytically active form of the complex ion is either an RNA-bound metal hydroxide that acts by abstracting a proton from the 2′-hydroxyl at the cleavage site (an outer-hydration-sphere mechanism), or a metal ion bound directly to the active site 2′-hydroxyl that causes the 2′-proton to ionize (an inner-hydration-sphere mechanism). The cleavage reaction then proceeds via an 'in-line' or $S_N2(P)$ mechanism, as described in the previous section. The rate of divalent metal ion-assisted catalytic cleavage generally increases with decreasing pK_a of the metal hydroxide. This observation has been used to suggest that the active species is indeed a metal hydroxide (Dahm *et al*. 1993), but this interpretation has been challenged by Pontius *et al*. (1997) who

point out that metal hydroxides with lower pK_a values will be correspondingly weaker bases and therefore less able to abstract the 2′-hydroxyl proton, assuming the pK_a of the 2′-hydroxyl is higher. (The pK_a of the 2′-hydroxyl in a free nucleotide is about 12 or above, with two recent estimates placing this between 13·1 to 13·7 and at 14·9, respectively (Li & Breaker, 1999; Lyne & Karplus, 2000), and those of hydrated Mg^{2+}, Mn^{2+} and Cd^{2+} are 11·4, 10·6 and 9·6, respectively.) Although more of the metal hydroxide would be in the ionized state for hydrated divalent cations having lower pK_a values, the weaker Brønsted bases would be correspondingly less effective, such that the two effects would exactly cancel. Hence if a metal hydroxide was responsible for abstraction of the 2′-hydroxyl proton at the active site, and if this were part of a concerted reaction or the rate-limiting step of a sequential reaction, one would expect to see no correlation between cleavage rate and the pK_a values of the various metal hydroxides (Pontius *et al*. 1997).

The second potential opportunity for divalent metal ion-assisted catalysis is for a metal ion to interact directly with one of the nonbridging phosphate oxygens, in the manner of lysine, thus stabilizing the negative charges that accumulate in an oxyphosphorane transition-state structure. Replacing the *pro*-R phosphate oxygen at the active site with a sulfur reduces hammerhead catalytic activity in the presence of Mg^{2+}; this activity may be rescued partially by the addition of a softer (hence more thiophilic) divalent metal ion such as Mn^{2+}, indicating that Mg^{2+} (a relatively hard Lewis acid) binds directly to the *pro*-R oxygen at the cleavage site (Dahm & Uhlenbeck, 1991; Koizumi & Ohtsuka, 1991; Slim & Gait, 1991). Recently, this metal binding-site explanation has been questioned (Zhou *et al*. 1996a), based on the observations that substitution of a sulfur at the *pro*-S phosphate oxygen position at the cleavage site shows a similar Mn^{2+}-dependent rescue effect (Slim & Gait, 1991; Zhou *et al*. 1996a). Experiments using Cd^{2+} rather than Mg^{2+}, however, do indeed appear to be consistent with the originally proposed metal–*pro*-R phosphate oxygen interaction (Scott & Uhlenbeck, 1999). Cadmium is softer than magnesium, so the covalent character of the metal–sulfur bond will be enhanced further. This raises the question of whether by substituting a sulfur for an oxygen, one 'recruits' a metal that would not otherwise bind with high affinity. Nevertheless, binding of the softer cadmium ion preferentially rescues the sulfur substitution at the R position over the S position. A particularly intriguing result has been obtained with hammerhead RNAs that have *both* the *pro*-R and the *pro*-S nonbridging oxygens *simultaneously* substituted with sulfur atoms at the scissile phosphate. Unlike the single substitution of the *pro*-R oxygen with sulfur, which essentially abolishes the activity of the hammerhead ribozyme in the presence of Mg^{2+}, the phospho*di*thioate substitution at the cleavage site yields hammerhead ribozymes whose cleavage rates are relatively efficient (about 1000 times background rate) and are *not* rescued further by the addition of softer, more thiophilic ions such as Cd^{2+} (W. B. Derrick, C. Greef, M. Caruthers & O. C. Uhlenbeck, unpublished results). Given these results, it may in fact be that a single sulfur substitution in the *pro*-R position of the scissile phosphate simply creates a deleterious charge asymmetry that can be ameliorated either by restoring the charge balance with a phosphodithioate substitution or by the binding of a recruited thiophilic metal ion to the substituted sulfur.

A third potential opportunity for divalent metal ions to accelerate the hammerhead self-cleavage reaction is acid stabilization of the 5′-bridging oxygen leaving group as the scissile bond breaks. This can in principle be accomplished either by protonation of the 5′-oxygen as negative charge begins to accumulate (a form of general Brønsted acid catalysis) or by direct coordination (Steitz & Steitz, 1993) of the 5′-oxygen with a divalent metal ion such as

Mg^{2+} (Lewis acid catalysis). The Brønsted acid catalysis scheme is again an outer-sphere mechanism, and the Lewis acid mechanism is inner-sphere. The inner-sphere and outer-sphere mechanisms are actively disputed, (Taira *et al*. 1990; Kuimelis & McLaughlin, 1995; Sawata *et al*. 1995; Zhou *et al*. 1996b; Pontius *et al*. 1997; Lott *et al*. 1998), based upon the lack of an observable thiophilic metal ion rescue of a hammerhead RNA substrate that has the 5′-bridging oxygen of the scissile phosphate substituted with a sulfur atom (Kuimelis & McLaughlin, 1995; Zhou *et al*. 1996b). In addition, a solvent-isotope effect of $k_{obs}(H_2O)/k_{obs}(D_2O) = 4$ has been invoked to propose that there cannot be a proton transfer in the rate-limiting step of the reaction (Sawata *et al*. 1995), although this interpretation, too, has been challenged (Pontius *et al*. 1997). Finally, the results of experiments using micromolar quantities of La^{3+} to enhance and subsequently inhibit the cleavage rate of the hammerhead ribozyme in a constant millimolar background of Mg^{2+} have been offered as further evidence that two metal ions participate in the chemistry of the cleavage reaction by forming inner-sphere complexes to the 2′-oxygen and the 5′-oxygen in the course of the reaction (Lott *et al*. 1998). In this experiment, maximum activity was observed when 3 μM La^{3+} was added to 8 mM Mg^{2+} in 200 mM NaCl at pH 7 in reaction mixtures using hammerhead 16; additional La^{3+} inhibited the reaction. These data were used to propose that two Mg^{2+} ions, one that binds directly to the 2′-oxygen, allowing the 2′-proton to dissociate more readily, and a second that binds directly to the 5′-oxygen, allowing accumulating negative charge to be absorbed, bind with K_d values of 3·5 mM and > 50 mM, respectively. The authors further argue that these observations can only be consistent with a two-metal-ion mechanism in which both metal ions directly coordinate their oxygen ligands *via* inner-sphere interactions (Lott *et al*. 1998). Possible transition states corresponding to the three different reaction mechanisms are shown in Fig. 3.

The structural role of divalent metal ions has also been investigated by way of several independent experimental techniques, including gel electrophoretic mobility, fluorescence resonance energy transfer (FRET), NMR and X-ray crystallography. The NMR and crystallographic results will be discussed in Section 5. Here we will consider the conformational dynamics of the hammerhead ribozyme as revealed by electrophoretic mobility (Bassi *et al*. 1996, 1997) and FRET analyses (Tuschl *et al*. 1994; Bassi *et al*. 1999). In low ionic strength conditions with 10 mM Mg^{2+} present, gel electrophoretic mobility experiments (Bassi *et al*. 1996, 1997), transient electric birefringence experiments (Amari & Hagarman, 1996), FRET experiments (Tuschl *et al*. 1994; Bassi *et al*. 1999) and X-ray crystallographic experiments (Scott *et al*. 1995) all appear to yield results consistent with a folded hammerhead RNA molecule in which Stem II is extended by noncanonical base-pairings of conserved residues and stacks approximately coaxially upon Stem III, and Stem I forms an acute angle with Stem II. (The details of the crystal structures are described in Section 5.) When only 0·5 mM Mg^{2+} is present, however, the RNA appears to unfold partially, as observed by electrophoretic mobility and FRET, such that Stem I now appears to form an acute angle with Stem III (Bassi *et al*. 1996, 1997, 1999). At low ionic strength when Mg^{2+} is completely absent, the hammerhead RNA appears to be completely unfolded, where electrophoretic mobility and FRET results are consistent with an extended structure such as that depicted in the canonical secondary-structure representation (Fig. 1(*a*)) that looks like a hammerhead (Bassi *et al*. 1996, 1997, 1999). These results strongly imply that two different Mg^{2+} under standard reaction conditions are responsible for allowing the hammerhead RNA to fold correctly prior to catalysis. These structural Mg^{2+} ions appear to bind with estimated

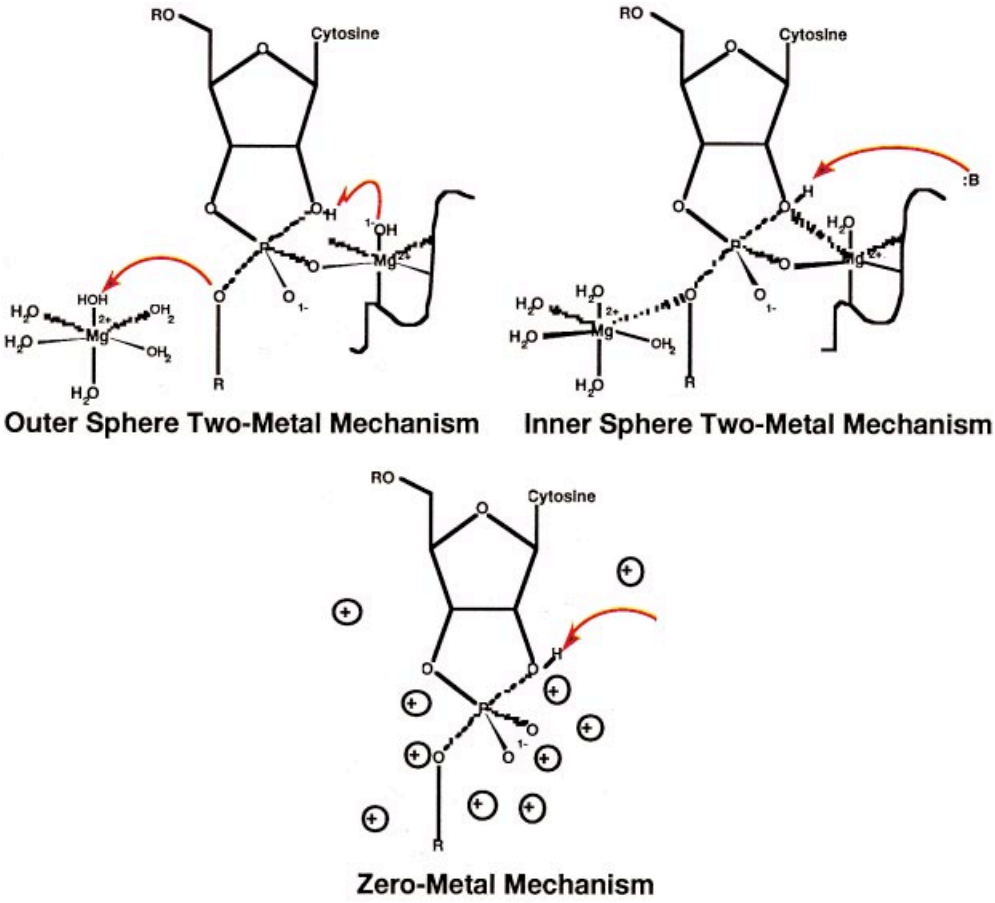


Fig. 3. Possible transition states corresponding to three different reaction mechanisms. The first (left) is a reaction mechanism where two hydrated divalent metal ions, one functioning as a Brønsted base (in the metal-hydroxide form) abstracts the 2′-proton, and the other, a Brønsted acid, donates a proton. The first metal is also shown directly coordinated to the *pro*-R oxygen, although this coordination can be by the second or even a third divalent metal ion. The second (middle) is a reaction mechanism wherein one divalent metal ion directly coordinates the 2′-oxygen, thus lowering the effective pK_a of the 2′-hydroxyl, and the other divalent metal ion, acting as a Lewis acid catalyst, directly coordinates the 5′-oxygen as negative charge begins to accumulate at that atom. The third reaction mechanism (right) is not metal dependent, but is simply electrostatic in character. The positive charges can be supplied at high density either in the form of divalent cations (similar to the second reaction mechanism) or by any cation at sufficiently high local concentration. The charges are shown arranged nonspecifically to emphasize that such a mechanism is not dependent upon the existence of a specific binding site or pocket.

K_d values of approximately 100 μM and 1 mM (Bassi *et al*. 1999). The authors suggest that the folded structure might then create binding sites for the catalytic metal ions to then occupy.

3.3 The hammerhead ribozyme does not actually require metal ions for catalysis

Because of the volume of research devoted to understanding the mechanistic roles of divalent metal ions in hammerhead ribozyme catalysis, and because a fundamental tenet of ribozyme enzymology has been that all ribozymes are metallo-enzymes, it was somewhat surprising to

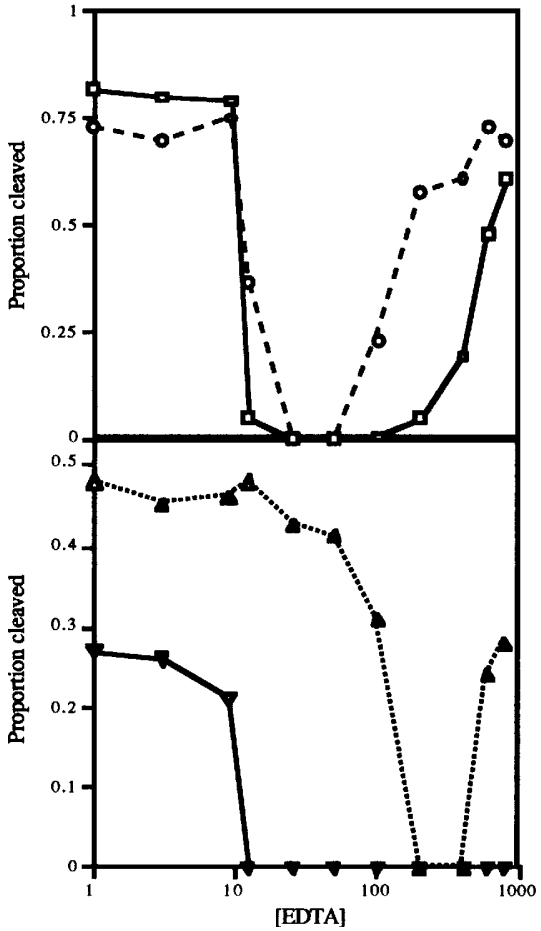


Fig. 4. Na_3EDTA titrations demonstrate that magnesium-dependent ribozyme-catalyzed RNA cleavage reactions of the $HH_{16\cdot1}$ (□), Hairpin (○) and VS (△) ribozymes but not the HDV ribozyme (▽) are quenched by EDTA and stimulated by monovalent cations (Murray *et al*. 1998b).

find that at least three of the four small, naturally occurring ribozymes can function reasonably efficiently in the absence of divalent metal ions, providing that very high concentrations of monovalent cations (i.e., 4 M Li^+ or even 4 M NH_4^+) are present (Murray *et al*. 1998b). This is dramatically illustrated in Fig. 4, which shows that EDTA can abolish cleavage activity by sequestering divalent cations, as one would expect, but in the cases of the hammerhead, hairpin and *Neurospora* VS hammerheads (i.e., three of the four naturally occurring small self-cleaving RNAs), the activity returns when the concentration of EDTA, and therefore Na^+, is increased further. High concentrations of Li^+, Na^+, NH_4^+ and other monovalent cations apparently enable the RNA to fold in much the same way that divalent metal ions allow it to. (The crystal structures of the hammerhead ribozyme in the presence of 1·8 M Li_2SO_4 and in the presence of 10 mM $MgCl_2$ at low ionic strength are identical within experimental error.) It therefore appears that RNA folding accounts for much, if not all, of the catalytic enhancement over background rates found with these ribozymes. For example, hammerhead 16·1 (Clouet-d'Orval & Uhlenbeck, 1997), which is considered to be an optimized hammerhead ribozyme sequence for single-turnover reactions, cleaves only three-

fold faster in the presence of 10 mM $MgCl_2$ and 2 M Li_2SO_4 than it does in the presence of 2 M Li_2SO_4 alone. The rates of hairpin and VS ribozymes in 2 M Li_2SO_4 actually exceed those measured under 'standard' low ionic strength conditions (Murray *et al*. 1998b), and the rate of cleavage for the non-optimized hammerhead sequence used for crystallization is fivefold enhanced in 2 M Li_2SO_4 alone versus standard reaction conditions (Murray *et al*. 1998a). The nonoptimized sequence used for crystallization tends to form alternative, inactive structures in solution, such as a dimer of the enzyme strands, that dominate at lower ionic strength.

It has been objected that 2 M Li_2SO_4 is hardly physiological, and therefore that the lack of a requirement for divalent metal ions is an artificial one, likely an *in vitro* artifact. Although this may be the case, a similar line of reasoning then must lead us to the conclusion that catalysis by Group I introns and bacterial RNase P must be *in vitro* artifacts. Group I introns and bacterial RNase P function as RNA–protein complexes *in vivo*. The discovery that RNA can be catalytic (Kruger *et al*. 1982; Zaug & Cech, 1986) involved isolating the RNA components of these complexes *in vitro* and providing an environment of suitable ionic strength to compensate for the lack of the protein components. Under these *in vitro* conditions, which are also nonphysiological, the Group I intron RNA and the bacterial RNase P RNA can function as catalysts. But they appear to require their protein components to fold correctly and therefore to be catalytic *in vivo*. Is RNA catalysis in general therefore an *in vitro* artifact?

The importance of the discovery of catalytic RNA is that the protein components of these complexes are not *fundamentally* required for catalytic (or enzymatic) activity. They appear to play an ancillary structural role rather than a direct chemical role in ribozyme catalysis, and their apparent *in vivo* requirement can be substituted for *in vitro* by inclusion of nonphysiological concentrations of various salts. Similarly, metal ions are not *fundamentally* required for hammerhead, hairpin or *Neurospora* VS ribozyme catalysis, even if these catalytic RNAs rely on the presence of physiological concentrations of Mg^{2+} *in vivo*, because, like the protein components of the larger ribozymes, one can find *in vitro* conditions in which the Mg^{2+} is not required for catalysis. In the case of the hairpin ribozyme, aminoglycoside antibiotics and spermine have been found to accelerate the self-cleavage reaction in the absence of divalent cations, again suggesting that these polycations somehow substitute for the ancillary structure role played by divalent metal ions in the case of this ribozyme (Earnshaw & Gait, 1998).

We are therefore left with two possible outcomes in this analysis. The first outcome must dismiss the relevance of catalytic RNA on the same logical grounds that it dismisses the lack of a metal ion requirement. The second outcome acknowledges that RNA can indeed be an enzyme, and that hammerheads do not necessarily have to be metalloenzymes. I believe the second point of view is preferable because it not only recognizes the obvious importance of catalytic RNA, but also reminds us that it is the RNA molecule that is actively catalytic, and is not simply an elaborate but largely inert structure of ancillary importance designed for binding catalytic metal ions in proximity to a scissile phosphate (Scott, 1999).

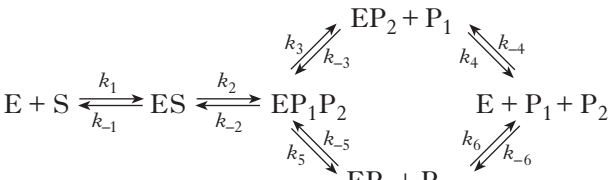
At this point the safest conclusion is that divalent metal ions likely assist hammerhead RNA assembly under physiological conditions but are not *fundamentally* required to do so. This is a situation much like that observed with tRNA. It may also be the case that divalent metal ions participate in the cleavage reaction chemistry when present, and may even do so in a variety of different ways depending upon the species of divalent metal ion present, but again are not a *fundamentally* required participant in the cleavage chemistry. Either they are

dispensable entirely, or they can be mimicked rather efficiently by any locally high concentration of positive charge whose chemical identity is not critical. If the latter conclusion is correct, it suggests that the reaction mechanism is primarily electrostatic (a 'hard–hard' acid–base reaction), but perhaps can be co-opted by one more covalent in character (a 'soft–soft' acid–base reaction artificially created by phosphorothioate substitution and recruitment of a soft Lewis acid). The suggestion that the reaction must be primarily electrostatic is perhaps the only interpretation that can be reconciled with the results and interpretation of Lott *et al*. (1998) described above, in that inner-sphere interactions between Mg^{2+} (a hard Lewis acid) and the 2′-oxygen or the 5′-oxygen are likely to be primarily electrostatic. The other alternative is that Lott *et al*. (1998) have observed binding of the two Mg^{2+} ions consistent with Mg^{2+} ion-induced structural transitions that take place in the course of assembly of the hammerhead ribozyme observed by fluorescence resonance energy transfer experiments, as discussed in the previous section, and that these ions are not actually involved directly in the chemistry of ribozyme catalysis. The similarity of the two observed K_d values for Mg^{2+} and the Mg^{2+} concentrations at which the two structural transitions occur (see below) is particularly striking. Such an interpretation has been hinted at in the case of the metal ion associated with the ionizing 2′-hydroxyl (Pontius *et al*. 1997). It is also noteworthy that in the case of the 5′-S-substituted leaving group hammerhead ribozyme substrate, where the leaving group no longer requires stabilization as noted above, the hammerhead cleavage reaction is spontaneous even in the absence of divalent metal ions under standard reaction conditions and with spermine present to aid folding the ribozyme (Kuimelis & McLaughlin, 1995). Taken together, these considerations permit suggestion that catalytic enhancement in the case of the hammerhead ribozyme self-cleavage reaction takes place primarily at the site of the leaving group, that bond-scission is the rate-limiting aspect of the reaction, and that all that is required for catalysis, fundamentally, is the presence of a positive charge at high local concentration to balance the accumulating negative charge on the 5′-oxygen as the bond is cleaved. It is noteworthy that this proposal is also consistent with the observed metal ion pK_a dependence of the cleavage reaction rate (Pontius *et al*. 1997) as well as the lack of a thio-effect in the cleavage-site phosphodithioate hammerhead substrate mentioned earlier (W. B. Derrick, C. Greef, M. Caruthers & O. C. Uhlenbeck, unpublished results).

3.4 Hammerhead RNA enzyme kinetics

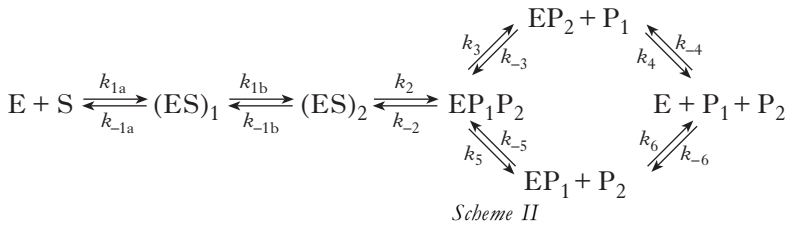
Much effort has been devoted to elucidating the kinetic properties of the hammerhead ribozyme. Under 'standard' reaction conditions, the hammerhead ribozyme exhibits simple Michaelis–Menton enzyme kinetics, meaning that in the limit of negligible product concentration and rapid dissociation of the cleavage products, the reaction can be characterized simply as an association of enzyme and substrate to form an enzyme–substrate complex followed by catalytic turnover. Typical K_m values for the reaction are on the order of micromolar or less, implying that the helical dissociation equilibrium tends to dominate K_m. Typical k_2 values are on the order of 1 turnover/minute under standard reaction conditions at pH 7·5, but this can be as much as 10-fold higher for one particular sequence that has not been observed in nature, but was instead discovered in the laboratory (Clouet-d'Orval & Uhlenbeck, 1996). Because of the relatively slow turnover rate, perturbations of the hammerhead reaction mechanism that primarily affect K_m have been descried as 'ground-

state effects', while those that affect k_2 have been described as 'transition-state effects', as will be described below. Depending upon the sequence of the hammerhead ribozyme under consideration, product dissociation rates can be significant, and a more generally valid minimal reaction scheme has been proposed in which the two product strands dissociate in a random bimolecular manner (Hertel *et al*. 1994). This scheme, as well as others that involve possible conformational changes within the enzyme–substrate complex under 'standard' (10 mM $MgCl_2$ at pH 7·5) reaction conditions, are described in a comprehensive review of hammerhead ribozyme enzyme kinetics (Stage-Zimmermann & Uhlenbeck, 1998).



Scheme I

Recalling that steady-state enzyme kinetics experiments are incapable of distinguishing between a single enzyme–substrate complex and a series of enzyme–substrate complexes in conformational equilibria, it is possible that the minimal reaction scheme actually contains two or more species of enzyme–substrate complexes. This is of particular relevance in the context of the hammerhead ribozyme crystal structure, as described in detail in Section 5.4. Briefly, if the crystal structure represents an 'on-pathway' enzyme–substrate complex, then it is required to undergo a conformational change to bring the scissile phosphate into a conformation amenable to an 'in-line' attack mechanism. Therefore, if the crystal structure represents $(ES)_1$, there must exist another structure, $(ES)_2$, prior to the chemical step of the reaction. The only other possible alternatives are that the crystal structure represents an 'off-pathway' conformation, or that the observed requirement for an 'in-line' mechanism is flawed. Barring such alternatives, a minimal scheme that includes the initial-state crystal structure as $(ES)_1$ and the conformation amenable to in-line attack as $(ES)_2$ can be written as follows:



Scheme II

The numbering of the rate constants has been chosen in such a way as to emphasize that the equilibrium of Step 1 in the first scheme may be an indistinguishable composite of two consecutive equilibria (1a and 1b) in the second scheme under steady-state conditions. Of course, there may actually be even more than two enzyme–substrate complex conformations on the reaction pathway, requiring an even more complex kinetic mechanism. This in fact appears to be the case when the hammerhead ribozyme is characterized by electrophoretic

mobility or fluorescence resonance energy transfer (FRET) experiments under various concentrations of divalent cations. According to these studies, $(ES)_1$ in Scheme II should be replaced by three separate species in sequential equilibrium, i.e.,

$$E + S \underset{k_{-1a'}}{\overset{k_{1a'}}{\rightleftharpoons}} (ES)_{1'} \underset{k_{-1a''}}{\overset{k_{1a''}}{\rightleftharpoons}} (ES)_{1''} \underset{k_{-1a'''}}{\overset{k_{1a'''}}{\rightleftharpoons}} (ES)_{1'''} \underset{k_{-1b}}{\overset{k_{1b}}{\rightleftharpoons}} (ES)_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \cdots$$

Scheme III

where $(ES)_{1'}$ is the dominant species under low ionic strength conditions in the absence of divalent cations, and is believed to be an extended from of the enzyme–substrate complex that resembles the canonical secondary structure in which the Watson–Crick helices have formed but the core region is disordered, where $(ES)_{1''}$, a partially assembled folding intermediate, is the dominant species in the presence of approximately 0·5 mM Mg^{2+}, and $(ES)_{1'''}$ is the dominant species in the presence of 10 mM Mg^{2+} and is thought to be the same as the initial-state crystal structure, or $(ES)_1$ in Scheme II, based on FRET measurements and analyses (Bassi *et al*. 1996, 1997, 1999). Since Scheme II appears to represent the minimal kinetic mechanism under 'standard' reaction conditions, it is likely that the folding sequence in Scheme III is quite rapid, or perhaps concerted, when 10 mM Mg^{2+} is present.

Because standard steady-state and even pre-steady-state single-turnover experiments are incapable of dissecting out multiple intermediates, other techniques have been employed to try to uncover the proposed structural rearrangement step (k_{1b} in Schemes II or III) prior to cleavage. One of these is cryo-enzymology (Feig *et al*. 1998), and the other is X-ray crystallographic intermediate trapping, discussed in Section 5.5. Within the confines of Scheme II, the equilibrium between $(ES)_1$ and $(ES)_2$ is believed to favor $(ES)_1$ strongly as the major precatalytic hammerhead ribozyme conformation, based upon the initial-state crystal structure and solution NMR results. The authors of the cryoenzymology study (Feig *et al*. 1998) also propose that both k_{1b} and k_{-1b} must be quite fast compared to k_2 in order for the equilibrium between $(ES)_1$ and $(ES)_2$ to be unobserved kinetically. Although both rates will likely be temperature dependent, it is possible that their temperature dependencies will differ significantly enough to find conditions in which $k_2 > k_{1b}$, so that the formation of $(ES)_2$ from $(ES)_1$ becomes rate limiting and can then be observed. The normal rate-limiting step is assumed to be the chemical step (k_2) based upon its pH dependence.

In the case of protein enzymes, it has been possible to find low temperature regimes in which enzyme–intermediate complexes can be detected kinetically in appropriate cryosolvents (Fink & Geeves, 1979; Fink & Petsko, 1981). In practice, this is manifested in a biphasic (or multi-phasic) Eyring plot ($\ln k_{obs}$ vs. $1/T$) that indicates a transition from one rate-limiting step to another in the kinetic mechanism as a function of temperature. In the case of the hammerhead ribozyme in 40% methanol, the reaction observed at room-temperature in aqueous solutions is maintained down to −27 °C. Below this temperature, an abrupt reduction in activity takes place. Both phases of the Eyring plot are linear but have sharply differing slopes, although the lower-temperature phase could only be measured between −27 °C and −33 °C. Both phases also showed a pronounced dependence of the reaction rate upon pH. There are at least three explanations for the observed biphasic Eyring plot: (1) the sought-after conformational change becomes rate-limiting; (2) the RNA undergoes a glassy transition in which it loses the elastic properties required to bind the substrate; or (3) the RNA undergoes a cold-denaturation transition. The authors believe that the first explanation,

i.e., that they have observed a pre-catalytic conformational intermediate, is unlikely because they do not expect such a conformational change to be strongly pH dependent, and that the second explanation is not likely because the glassy-transition temperature for protein enzymes is much lower (−60 °C to −70 °C). This leaves cold-denaturation as a possible explanation for the biphasic Eyring plot the most likely explanation, unless evidence that the conformational change is significantly pH dependent emerges. (This possibility will be considered further in Section 5.8.)

4. Sequence requirements for hammerhead RNA self-cleavage

4.1 The conserved core, mutagenesis and functional group modifications

The hammerhead RNA sequence motif consists of three base-paired stems flanking a central core of 15 conserved nucleotides, (see Fig. 1(*a*) above). The numbering scheme for the helices and bases shown in Fig. 1(*b*) has been standardized (Hertel *et al*. 1992). The 15 conserved central bases, shown as outlined letters, are essential for ribozyme activity (Ruffner *et al*. 1990). Nine of these conserved bases cannot form conventional Watson–Crick base-pairs, but instead form more complex structures that mediate RNA folding and catalysis. Substitution of any of the nominally unpaired conserved bases with other naturally occurring bases, or sometimes even artificial alteration of their functional groups, results in significantly diminished catalytic activity (Thomson *et al*. 1996; McKay, 1996). In addition, two sets of base-pairs in Stem III and one pair in Stem II are conserved; changing these to other base-pairs either impairs (in the case of the 15·2–16·2 and 10·1–11·1 pairs) or abolishes (in the case of the 15·1–16·1 pair) catalytic function. A comprehensive summary of mutations and functional group alterations has been published in a recent review (McKay, 1996). Some of these observations are summarized in the following paragraphs.

The conserved nucleotides in Stem III are an A-15·1 paired to a U-16·1, an absolutely conserved pair, and a C-15·2 paired with a G-15·2, a preferred but not absolutely required pair. Both are predicted to form Watson–Crick base-pairs according to the secondary structure of the hammerhead RNA, although the crystal structures (see below) reveal an unusual hydrogen bonding scheme in which only one hydrogen bond, between the exocyclic amine of A-15·1 and O4 of U-16·1 is present, and an additional hydrogen bond forms between the exocyclic amine of A-15·1 and the exocyclic oxygen of G-16·2, suggesting the preference for the GC pair is to stabilize the unusual AU geometry via an additional hydrogen bond in a bifurcated base-pairing scheme (Scott *et al*. 1995). The O4 of U-16·1 is strictly required (Murray *et al*. 1995), but removal of the exocyclic amine from A-15·1 is somewhat more tolerated (Slim & Gait, 1992; Fu *et al*. 1993).

The conserved nucleotides in Stem II are G-10·1 and C-11·1. They form a conventional base-pair, but 10·1 has in addition a metal-binding site associated with its N7 as observed in the original crystal structure (Pley *et al*. 1994). Switching the orientation of this base-pair, or changing it to anything except a U–U pair, is quite inhibitory. It is likely that a U–U pair can also function as a metal binding site.

The remaining nine conserved nucleotides are not predicted to form canonical base-pairs on the basis of the sequence of the hammerhead ribozyme, and indeed none are found in the crystal structure. All but two of these nine remaining conserved nucleotides are purines, and

all nine are completely intolerant to nucleotide substitution (Ruffner *et al*. 1990). For that reason, mutations that consist of unnatural (or rare) bases that have a few or only one of the functional groups changed have been studied intensively (see McKay, 1996, for a comprehensive tabular summary) with the hope of identifying specific hydrogen bonding patterns that eluded identification in terms of nucleotide covariant substitutions (Ruffner *et al*. 1990) analogous to those used to pinpoint the identity of base-triples in tRNA (Levitt, 1969). These experiments for the most part did not reveal hydrogen bonding schemes as unambiguously as had been anticipated, sometimes tended to contradict one another, and were often in conflict with the observed hydrogen bonding pattern seen in the crystal structure (McKay, 1996; Wedekind & McKay, 1998) as described below, indicating that either they, or the crystal structure, must be problematic.

The most striking trends are that alteration of any of the three Watson–Crick hydrogen bond donor or acceptor functional groups on any of the three guanosines in the conserved core region is highly deleterious to the ribozyme's catalytic activity, and that in contrast, alteration of any exocyclic amine group on any of the five adenosines in the conserved core and Stem II results in only a slight to moderate loss of activity (McKay, 1996, cf. Fig. 2), as is the case with the exocyclic amine of C-3, C-17 and the O4 oxygens of U4 and U7. One possible interpretation of these data that has not been suggested previously is that this pronounced uniformity in responses may be a better predictor of the identity of a nucleotide than it is of hydrogen bonding patterns, and that guanosine in particular may be intrinsically more sensitive to alteration that are the other nucleotides. If this is the case, the utility of base functional group alteration studies for deducing RNA tertiary structure may have some rather serious limitations, at least in the context of the hammerhead RNA, since functional group alteration of guanosine appears to be too coarse in its effects to detect the presence of hydrogen bonds, and alterations of the other functional groups of A, C and U in general always have only slight effects (U-16·1 being the one exception), again limiting their usefulness. Alteration of the 2′-hydroxyls, on the other hand, is more revealing; only those of G-5 and G-8 (both observed to make hydrogen bonding contacts in the crystal structure), as well as that of the cleavage-site nucleotide (essential for catalysis), are strictly required.

4.2 Ground-state vs. transition-state effects

In general, the effects of a mutation will change either the k_m or the k_{cat} of the reaction, or will change both. Although K_m is a collection of rate constants, it can approximate the dissociation constant if k_{cat} is significantly smaller than the rate of enzyme–substrate association in a simple Michaelis–Menten scheme. For that reason, mutations that primarily affect K_m without significantly altering k_{cat} are often called 'ground-state effects', and mutations that primarily change k_{cat} without significantly disrupting the K_m are termed 'transition-state effects'. The former are equated with formation of the enzyme–substrate complex; the latter with interactions proposed to exist in the transition-state structure that are absent in the ground-state structure. These latter interactions presumably account for the transition-state stabilization thought to be the hallmark of enzymatic catalysis.

For example, removal of the exocyclic amine of G-5 results in a 250-fold decrease in k_{cat} while only increasing K_m sixfold (Tuschl *et al*. 1993). Loss of the exocyclic amine is thus interpreted to have a fairly small effect upon the formation of the enzyme–substrate complex, but a fairly profound effect upon the stabilized transition-state structure. Most of the other

functional group alterations summarized in McKay (1996) have even smaller effects upon K_m while in some cases (those of G-8 and G-12) the effects upon k_{cat} are much greater than the example cited with G-5 (see McKay, 1996). Again, the standard interpretation is that these mutations do not significantly disrupt the structural stability of the enzyme–substrate complex, but do disrupt the stability of the transition-state structure rather profoundly. These interpretations have lead to the assertion that the modification data are in conflict with the crystal structure, because in each case it is unclear from the ground-state crystal structure (see Section 5.1) how these functional groups participate in the transition-state structure. This assertion, in turn, has lead to the proposal that a global conformational rearrangement in the hammerhead ribozyme from that observed by X-ray crystallography must take place to form the transition-state structure. This proposal, along with its motivation and merits, will be discussed in the context of the crystal structure in Section 5 below.

The division between ground-state effects and transition-state effects relies upon an approximate transition-state theory in which the two species are regarded as isolated entities in equilibrium. This approximation is not strictly valid, as the two are more accurately described as states on a reaction coordinate that correspond to particular regions or points on a continuous Born–Oppenheimer potential energy surface. Motions or fluctuations associated with the enzyme–substrate complex state are therefore by necessity coupled to the transition state and vice versa. The theoretical basis for this assertion is described in detail in a recent review (Cannon *et al*. 1996); the net result of the approximate transition-state theory when applied to enzymology is a gross over-estimate of the tightness of binding of the transition state by the enzyme relative to the so-called ground state, leading to conclusions such as that changing the exocyclic amine of G-8 to a hydrogen-bond acceptor has the energetic cost of four very strong hydrogen bonds, conclusions that are difficult to rationalize physically in many cases.

The other assumption implicit in such interpretations is that a simple Michaelis–Menten scheme applies, whereas it seems more physically reasonable that K_m (or more accurately, K_d) reflects the binding of the enzyme and substrate strands in the canonical base-pairing regions as depicted in scheme III, i.e., that the K_m only reflects formation of the canonical secondary structure. If so, effects upon 'k_{cat}' may in reality actually reflect structural perturbations in the tertiary interactions that stabilize one of the intermediate structures in Scheme III rather than (or in addition to) the transition state.

5. The three-dimensional structure of the hammerhead ribozyme

5.1 Enzyme–inhibitor complexes

How does the three-dimensional structure of an RNA enzyme enable its catalytic activity? To answer this question, two research groups (Pley *et al*. 1994; Scott *et al*. 1995) crystallized the hammerhead ribozyme and determined its structure by using X-ray crystallography. The first structure was of a hammerhead RNA enzyme strand bound to a RNA substrate analogue (Pley *et al*. 1994) and the next was of an all-RNA hammerhead ribozyme with a 2′-O-methylated cleavage-site base modification (Scott *et al*. 1995); both approaches were designed to prevent catalytic turnover in the presence of divalent metal ions in the crystallization mixtures. The RNA folds in the catalytic cores of these hammerhead RNA structures were very similar to one another, despite several significant differences in approach, suggesting that

the observed RNA fold was not an artifact of crystallization. However, some concern remained that the modifications each group employed to prevent cleavage might have somehow similarly distorted the two structures.

The main features elucidated from these crystal structures are summarized in Fig. 1(*b*), where the enzyme strand (as with Fig. 1(*a*) and 1(*c*)) is shown in red, the substrate strand in yellow, and the cleavage-site base (C-17) is shown in green. In particular, an absolutely conserved four-nucleotide loop, having the same sequence (CUGA) and structure as the uridine turn found in $tRNA^{Phe}$ (Pley *et al*. 1994), forms a catalytic pocket (Scott *et al*. 1995) into which the cleavage site base, C-17, is inserted. This region of the structure is also known as 'Domain I' (Pley *et al*. 1994), although it is unclear if this RNA motif constitutes an autonomous fold, as with protein domains. The remainder of the ribozyme, including the conserved residues that augment Stem II, (also known as 'Domain II') apparently serves several structural roles that include mediation of a three-strand junction (as described below), and positioning the cleavage-site base into the catalytic pocket. The catalytic pocket itself presumably facilitates conformational rearrangements required for catalysis.

The global conformation of the all-RNA hammerhead ribozyme is depicted in Fig. 1(*c*) (above) as a roughly γ-shaped fold. Stem II and Stem III are approximately coaxial, with Stem I and the catalytic pocket branching away from this axis. Stem II, augmented by two GA reversed-Hoogsteen base-pairs and an unusual AU base-pair (collectively known as Domain II), stacks directly upon Stem III, forming one pseudo-continuous helix. The helix is not actually continuous, because it incorporates a three-strand junction where the active site cytosine (C-17, shown in green) is displaced from the helical stack and is instead positioned into the four-nucleotide catalytic pocket or Domain I. This pocket is formed by a sharp turn in the hammerhead enzyme strand – the uridine turn – that forms a very rigid structure in the crystal and is one of the most prominent features in the original MIR electron density maps. The phosphate backbone strands that diverge at the three-strand junction subsequently reunite to form Stem I. These structural features are illustrated schematically in Fig. 1(*b*), which is color-coded to complement Fig. 1(*a*) and 1(*c*) (Scott *et al*. 1995).

The crystal structures also revealed some features of metal binding to the hammerhead ribozyme. In the first structure (Pley *et al*. 1994), several divalent metal ions were observed to bind essentially identically to the augmented Stem II helix. These divalent metal ions included Mn^{2+}, which is coordinated directly to the N-7 of G-10·1 (immediately explaining the strong preference for a G–C pair at this position and in this orientation) and the *pro*R oxygen of the adjacent A-9 phosphate. This was the only metal ion binding site to be observed in the first crystal structure, and is about 20 Å from the scissile phosphate. The second structure (Scott *et al*. 1995) revealed several presumed Mg^{2+} ions throughout the ribozyme. The strongest site was the most prominent feature in th original MIR maps, even before noncrystallographic symmetry averaging had been applied. This metal ion was closer to the cleavage site, but appeared to bind via the first solvation shell to two adjacent G–C base-pairs in a nonconserved region of the minor groove of Stem I. This site was therefore thought to be an artifact of the particular sequence chosen for crystallization, although subsequent electrostatic potential calculations seem to suggest that this region of Stem I may in general attract solvated divalent cations in a sequence-independent manner (Herman *et al*. 1997a, b).

Based upon the position of another $Mg(H_2O)_6^{2+}$ complex ion that was found near the catalytic pocket of the hammerhead ribozyme, and upon the similarly situated metal binding

site in the uridine turn of $tRNA^{Phe}$, we initially proposed a mechanism in which the $Mg(H_2O)_6^{2+}$ complex ion first 'docks' in the catalytic pocket by interacting with C-3 and C-17, and then would be drawn in toward the cleavage site 2′-hydroxyl group, as the base of C-17 changed position to stack upon A-6 and G-5, until it would be within striking distance. (The trajectory and final position of the complex ion were both inferred from the metal binding positions in the uridine turn of $tRNA^{Phe}$.) We originally suggested that the metal ion was not correctly bound to the 2′-hydroxyl of C-17 because of the interfering presence of the 2′-O-methyl substitution (Scott *et al*. 1995). We no longer believe that this is the case, as the crystal structure of the unmodified (catalytically active) ribozyme, described in Section 5.2, did not bind this metal differently. Hence it is unlikely that our original mechanistic proposal, which relied upon movement of the divalent metal ion, was correct, although the idea of the base of C-17 moving in such a way as to stack upon A-6 (suggested by A. Klug) appears to have been correct (even if the details of the model proposed were not), as will be described in Section 5.6 and 5.7.

5.2 Enzyme–substrate complex in the initial state

To test the hypotheses that (a) the RNA might be in an inactive conformation, and (b) that the metal might be bound in an aberrant manner, both due to the presence of the substitution at the cleavage site, we crystallized an unmodified, catalytically competent hammerhead ribozyme. The RNA was of the same sequence, but the crystallization conditions (1·8 M Li_2SO_4 at pH 5·5 with no divalent metal ion present) and crystal form were new, requiring that the structure be solved by molecular replacement. The new crystals revealed that the unmodified hammerhead RNA adopts the same conformation as before (despite using crystallization conditions that resulted in a new packing scheme), suggesting that the modification of the previous RNA did not induce a conformational change. By soaking divalent metal ions into these crystals at low pH, we also showed that the previous modification did not cause the metal ions to bind in an aberrant manner, refuting our original suggestion that metal-binding was altered by the presence of the 2′-O-methyl modification, and that the metal bound to C-3 and C-17 would drawn in toward the nucleophile to initiate cleavage.

5.3 Hammerhead ribozyme self-cleavage in the crystal

In addition to the new structural information, the crystals of unmodified, and therefore catalytically competent, hammerhead RNA enabled us to test for cleavage activity in the crystalline state. Since this crystal form is 78 % solvent, and since the hammerhead ribozyme is active in 1·8 M Li_2SO_4, it was possible to initiate the cleavage reaction by flooding the crystal with divalent cations while raising the pH using a soaking solution buffered at pH 8·5. In conditions in which $[Mg^{2+}] \leqslant 50$ mM and pH $\leqslant 8{\cdot}5$, the catalytic turnover rate in the crystal is approximately 0·4 molecules/minute. Under similar conditions in solution, this same hammerhead ribozyme construct, a sequence that was optimized for purposes of growing crystals rather than for catalytic prowess, cleaves at a rate of approximately 0·08 molecules/minute, permitting us to suggest that the crystal lattice is doing more to aid in the proper folding of the ribozyme than it is inhibiting its cleavage activity. Moreover, the extent of cleavage of the substrate in the crystal is complete, whereas the extent of cleavage of the

substrate in solution, even under single-turnover conditions in which the enzyme strand is present in excess, is often only 75–85% complete, even for the most kinetically 'well-behaved' and optimized (for single-turnover) hammerhead sequences such as hammerhead 16·1 (Clouet-d'Orval & Uhlenbeck, 1997).

It has been argued that the cleavage rate 'should' be on the order of 10 turnovers/minute (or even much higher) under these conditions in the crystal, and that for a sequence optimized for single-turnover catalysis such as hammerhead 16·1, this rate should exceed 100 turnovers/minute (Wang *et al*. 1999). It should be noted, however, that (a) this objection is based upon the assumption that the dependence of rate upon pH remains log-linear at pH 8·5, when in fact it begins to plateau in this region, (b) it assumes that a pH equilibrium has been established between the crystal and the soaking buffer, even though we have, using pH indicator dyes, shown that the pH in fact may be significantly lower inside the crystal, and (c) that all of the rate-dependence upon sequence can be explained away by the propensity of slower species to form alternative conformers, when the actual dependence is likely to be much more complex, and (d) that the RNA has less dynamic flexibility in the crystal, and that the lattice may indeed have an inhibitory effect with respect to a hypothetical pure state of properly folded molecules of this sequence in solution.

In addition to the enhancement in the cleavage rate and extent of cleavage upon this particular sequence of hammerhead RNA, it is also worth noting that there is the expected dependence of the rate of cleavage upon pH, and that several modifications in the cleavage site and G-5 that are deleterious in solution are at least as deleterious to cleavage activity in the crystal. Therefore it is unlikely that the crystallized hammerhead ribozyme cleaves via an aberrant mechanism.

5.4 The requirement for a conformational change

Although the crystal structures provide rationalizations for many of the previously reported experimental observations, several important problems remained unresolved (see, for example, McKay, 1996). These included the following three sets of discrepancies with the biochemical data.

(1) The scissile phosphate of both crystal structures (and in that of the unmodified RNA described in Section 5.2) is in a conformation compatible with an 'adjacent' rather than 'in-line' mechanism of nucleophilic attack on the phosphorus from the 2′-oxygen of the cleavage-site base (see Fig. 2). Yet the mechanism of cleavage observed independently by three research groups (van Tol *et al*. 1990; Slim & Gait, 1991; Koizumi & Ohtsuka, 1991), as described in Section 3.1, clearly indicates that hammerhead ribozyme cleavage takes place via an in-line mechanism.

(2) No metal ions (hydrated or otherwise) were observed within striking distance of the 2′-hydroxyl attacking nucleophile. Although a metal was observed in the region of the cleavage site as just described (Scott *et al*. 1995), it was not close enough to allow abstraction of the 2′-proton from the active-site hydroxyl. Instead, it was proposed that either the metal ion, or else the RNA, has to move to initiate cleavage. Alternatively, additional metal ions might bind to the RNA if it were in an unmodified form or as it approached the transition state, thereby assisting catalysis but evading detection by an X-ray crystallographic analysis of the enzyme–substrate–inhibitor complex.

(3) Several discrepancies between chemical modification data on the conserved core of the hammerhead RNA (described in Section 4) and the crystal structure could not be resolved. Most noteworthy of these is G-5 of the CUGA catalytic pocket; the exocyclic functional groups all appear to be critical for catalysis (as reviewed in McKay, 1996; Usman *et al*. 1996). Yet the base of G-5 in the crystal structures is not involved in hydrogen-bonding interactions with other parts of the hammerhead RNA. (It is, however, involved in important stacking interactions.) In addition, the other conserved guanosines, which reside in the augmented Stem II helix or Domain II, are also extremely sensitive to modification, as noted in Section 4.1, in terms of their effect upon the rate of catalysis but have little effect upon substrate binding. This has led to the suggestion that the functional groups on these bases may be critical only in the transition-state complex (Usman *et al*. 1996).

The need to bring the scissile phosphate into a conformation amenable to an in-line attack from the adjacent 2′-oxygen nucleophile can be taken as *prima facia* evidence for the requirement of a conformational change to bring the hammerhead RNA from the initial-state structure to the transition state for bond cleavage. In addition, the other apparent disagreements between the biochemical data and the initial-state crystal structure have been taken as further evidence that a large-scale conformational change must take place in order to arrive at a structure that involves the functional groups thought to be critical only in the transition state. There appears to be universal agreement that some sort of conformational change in the hammerhead ribozyme structure must take place for catalysis to occur. The extent of this change has, however, been the source of disagreement, as will be discussed in Section 5.10.

5.5 Capture of conformational intermediates using crystallographic freeze-trapping

To understand the nature of the proposed conformational change required to activate the hammerhead ribozyme for catalysis, and to determine the extent of this change, time-resolved crystallographic studies have been attempted with the aim of observing conformational intermediates preceding catalysis. These studies have been made possible by the fact that cleavage can be initiated by soaking crystals at an elevated pH in divalent cations, and that the rate of cleavage is slow (0·4 turnovers/minute) and can be controlled by adjusting the pH of the soaking solution. The time it takes for a fairly complex substrate (NADP) to diffuse into a crystal of isocitrate dehydrogenase measuring 0·5 mm in each dimension and to saturate the enzyme's active sites is approximately 10 s when measured directly by video absorbance spectroscopy (Stoddard & Farber, 1995). The corresponding time it takes a much smaller divalent metal ion to diffuse into and to saturate the considerably smaller hammerhead ribozyme crystals ($0{\cdot}3 \times 0{\cdot}25 \times 0{\cdot}25$ mm or smaller) is unlikely to be longer. Therefore, the diffusion time is sufficiently fast compared with the turnover rate to allow approximately synchronous initiation of the hammerhead ribozyme cleavage reaction throughout the crystal.

To initiate the reaction, a crystal can be soaked in the cleavage-activating solutions and subsequently removed from the solution and flash-frozen in liquid nitrogen or liquid propane to trap any accumulated intermediate state. Monochromatic X-ray data are then collected in the usual manner for cryoprotected crystals. This procedure, termed monochromatic crystallographic freeze-trapping, is regularly employed for capturing structural changes in proteins and protein enzyme chemical intermediates (Moffatt & Henderson, 1995). The

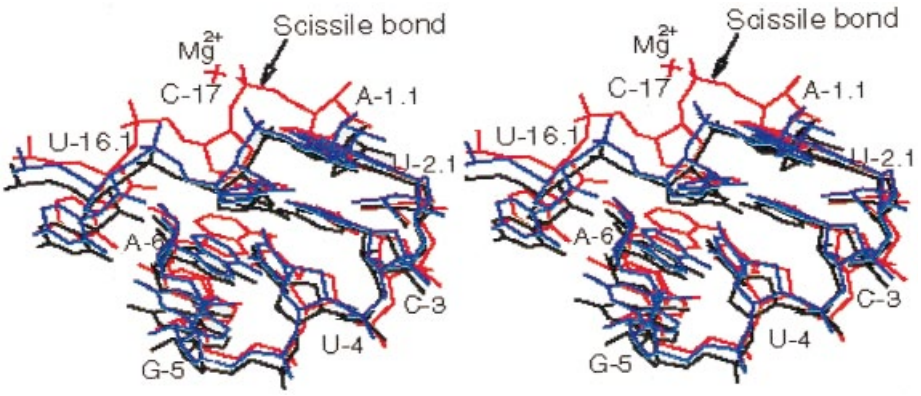


Fig. 5. A stereo view of the superposition of the catalytic pocket and adjoining bases from three crystals structures. The structure shown in red is the freeze-trapped conformational intermediate, the structure in blue is the 'ground-state' structure in the absence of divalent metal ions, and the black structure is the 2′-O-methyl-modified hammerhead ribozyme whose structure was previously determined in a different crystal form.

crystal may be removed from the X-ray source subsequent to data collection and stored frozen in liquid nitrogen until such time that assay of the extent of cleavage is convenient.

5.6 The structure of a hammerhead ribozyme 'early' conformational intermediate

Using the crystallographic freeze-trapping procedure, we captured the structure of a flash-frozen 'early' conformational intermediate that presumably exists only transiently in the crystal prior to cleavage under ordinary conditions. The most significant conformational changes were localized to the active site of the ribozyme, particularly to the scissile phosphate, which 'arches upward' by about 3 Å, as shown in Fig. 5. This conformational change is particularly suggestive, not only because it is the scissile bond that moves the most, but also because the new conformation requires only local adjustments to reach a proposed transition state compatible with an in-line attack mechanism (Scott *et al*. 1996). The 3 Å movement of the scissile phosphate in this 'early' intermediate could be detected readily and encouraged us to propose a mechanism for the hammerhead ribozyme cleavage reaction, invoking an *unobserved* more substantial movement of the scissile phosphate toward a conformation compatible with in-line attack. This crucial 'later' (and more chemically relevant) intermediate structure could not be captured using the unmodified hammerhead ribozyme crystals.

Binding of a divalent metal ion (Mg^{2+} or Mn^{2+} in subsequent experiments) in proximity of the scissile phosphate was observed to take place in the 'early' intermediate structure. This interaction appeared to be fascilitated by contacts to one of the nonbridging oxygen atoms in the scissile phosphate as well as to the N-7 of the nonconserved adenosine at position 1·1, the nucleotide just 3′ to the scissile phosphate (see Fig. 5). Based upon this observation, we speculated that this metal ion might be involved in catalysis, and would remain associated with the scissile phosphate throughout the conformational change or changes that preceded and included formation of the transition state, although it would likely be required to dissociate from A-1·1. Implication of this metal ion site as being critical for catalysis suffered from the flaw that binding to A-1·1 seemed to be required, whereas the most active hammerhead ribozymes contained a U at position 1·1, implying at the very least that such a

metal ion would need to bind in a different manner. The ultimate refutation of our hypothesis that the divalent metal ion moves with the scissile phosphate during a conformational change came as a subsequent observation (see below) that the 'later' conformational intermediate structure also showed clear metal ion electron density (this time Co^{2+}) in the original position (i.e., bound to the N-7 of A-1·1) even though the phosphate had moved in such a way that direct contact to the metal ion would no longer be possible. This observation, combined with Taira's finding that questioned the metal ion binding interpretation of the 'rescue' experiments, and later our own findings that divalent metal ions are not strictly required for catalysis, clearly indicates that the metal ion associated with the N-7 of A-1·1 is not likely to be a fundamental component of the self-cleavage reactions.

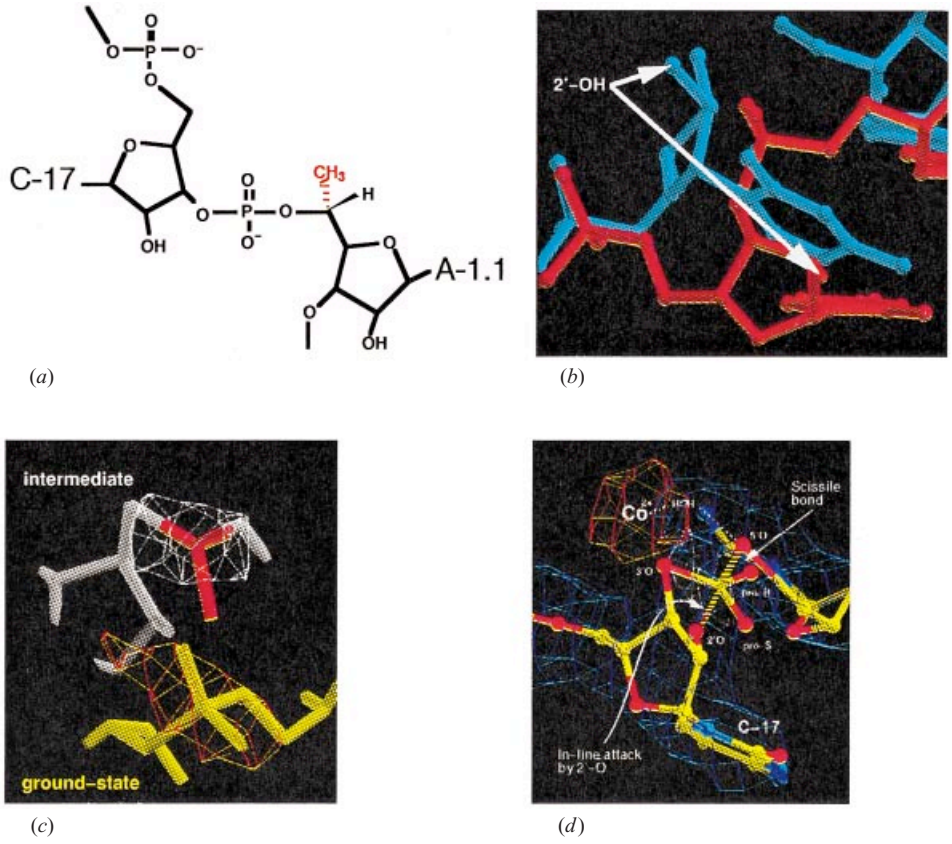
5.7 The structure of a hammerhead ribozyme 'later' conformational intermediate

To trap the structure of a 'later' hammerhead ribozyme intermediate, a hammerhead ribozyme having a 'kinetic bottleneck' at the final or bond-breaking point on the reaction pathway was synthesized using a modified leaving group. The idea for using such a modification was based upon the success of an analogous experiment with isocitrate dehydrogenase, in which active-site modifications allowed two intermediate structures each to accumulate in the crystal with artificially long life-spans, enabling their observation using polychromatic or Laue time-resolved crystallographic techniques (Bolduc *et al*. 1995).

By synthesizing a hammerhead ribozyme of the same sequence but with a *talo*-5′-C-methyl-ribose modification (Fig. 6(*a*)) designed to interfere with the stability or orientation of the leaving group of the reaction, a ribozyme was produced that has an unaltered attacking nucleophile, but a modified leaving group that inhibits the actual cleavage event (Beigelman *et al*. 1995; Beigelman, unpublished results). The additional methyl group stabilizes the ordinarily scissile bond between the cleavage-site phosphorus atom and the adjacent 5′-oxygen, presumably by altering the steric or electronic properties of this leaving group. This modified hammerhead RNA was used to capture a 'later' conformational intermediate that is poised to form an in-line transition-state.

Although the leaving-group-modified RNA has the same 'ground-state' and 'early-intermediate' structures as observed for the unmodified RNA, the modified RNA subsequently permitted capture of a 'later' and more informative intermediate structure in the course of the cleavage reaction. The base and ribose of C-17 in the later intermediate structure have rotated about 60° in such a way as to cause the base of C-17 to move over 7 Å to stack upon A-6 (which remains stacked upon G-5). Additionally, the furanose oxygen of A-1·1 now stacks upon the base of C-17, and therefore the entire platform of C-17, A-6 and G-5. Movement of the cleavage-site base has the effect of inducing a conformational change in the scissile phosphate, pulling the phosphate away from its original standard helical geometry, as shown by the difference Fourier peak for this new phosphate position in Fig. 6(*c*). The position of the omitted scissile phosphorus atom was subsequently deduced on the basis of difference Fouriers using phases calculated with the ground-state structure.

The 60° rotation of the base of C-17 with respect to the ground-state structure, combined with the concomitant conformational change of the C-17 ribose that flips the 2′-OH upward toward the scissile phosphate (Fig. 6(*b*)), has the effect of preventing the scissile phosphate from adopting the standard helical conformation that is found in all of the ground-state hammerhead RNA crystal structures, due to the geometrical restrictions created by the



(*a*) (*b*)

(*c*) (*d*)

Fig. 6. (*a*) The *talo*-5′-methyl-ribose modification of the hammerhead ribozyme leaving group; the added methyl group that slows the reaction at the bond-breaking step of the cleavage reaction about 100-fold is shown in red. This kinetic bottleneck allows capture of the conformational intermediate shown here. (*b*) A closeup of the cleavage-site base before (red) and after (light blue) the conformational change. Note the dramatic rearrangement in position of the 2′-OH. The scissile phosphate has been omitted from the intermediate structure at this point to eliminate model bias. (*c*) Another view of the initial (yellow) and intermediate (gray) structures, with the scissile phosphate of the intermediate modeled into positive difference density (white), and the initial-state phosphate occupying negative difference density in an $[F_{\text{interm}} - F_{\text{g.s.}}] \exp\{i(\Phi_{\text{g.s.}})\}$ difference Fourier. (*d*) The observed conformational change in the hammerhead ribozyme trapped intermediate structure is shown to be consistent with the future formation of an 'in-line' transition-state structure without any further major conformational changes of the ribozyme's three-dimensional structure.

movement of the cleavage-site base and ribose. This result is especially intriguing in light of the fact that the original helical phosphate conformation was highly incompatible with an in-line attack cleavage mechanism, as summarized in Fig. 2 and discussed in detail in Section 3.1. The effect of the C-17 conformational change is therefore to perturb the helical conformation of the scissile phosphate, thus rendering it more susceptible to nucleophilic attack from the cleavage site 2′-hydroxyl, also repositioned by the conformational change.

5.8 Is the conformational change pH dependent?

In Section 3.4, the results of a set of cryoenzymological analyses performed with the hammerhead ribozyme were summarized, along with the conclusions of the authors (Feig *et*

al. 1998). They observed a biphasic Erying plot, but because in both phases the cleavage reaction rate was pH dependent, they thought it was unlikely that they had isolated a genuine precatalytic conformational intermediate of the enzyme–substrate complex, since it was assumed that a conformational change of RNA should be pH independent. However, if the conformational change were in fact pH dependent, than it is possible that the authors actually did successfully observe a hammerhead ribozyme precatalytic intermediate.

Although it would be very difficult to prove that the 'late' conformational intermediate observed by X-ray crystallographic freeze-trapping, described in the previous section, was also detected by the cryoenzymological experiments, there is rather strong evidence that the earlier and later conformational changes observed by X-ray crystallography are both pH dependent. Specifically, the conditions under which cleavage in the crystal was activated included elevated pH (nominally to pH 8·5) in the presence of various divalent cations (Scott *et al*. 1996; Murray *et al*. 1998a). Subsequent to the discovery that divalent cations are not required for catalysis (Murray *et al*. 1998b), these experiments were repeated in the absence of divalent cations, and essentially the same conformational changes were again observed. This unambiguously demonstrates that the conformational changes observed by X-ray crystallographic freeze-trapping must be pH dependent. Moreover, these changes are not observed at pH 7, even when divalent cations (Mg^{2+} or Cd^{2+}) re present, even though the RNA still cleaves (albeit much more slowly). This again illustrates that the conformational changes must be stabilized by the higher pH. The higher pH environment likely stabilizes an intermediate structure that is less favorable at lower pH, and this intermediate structure, resembling the transition-state structure to an extent that is greater than the resemblance of the initial-state structure to the transition state, is likely closer to the catalytically active structure. Hence the intermediate accumulates in the kinetic-bottleneck experiment to a greater extent at high pH than it does at lower pH, and when the bottleneck is absent, the turnover rate is greater at high pH than it is at lower pH, in part because the intermediate is more stable at a higher pH.

Since the cleavage reaction is initiated when the 2′-proton is abstracted from the cleavage-site 2′-hydroxyl group, it is possible that the conformational change is driven by this ionization event, and that the resulting negative charge that appears on the 2′-oxygen attacking nucleophile can be stabilized if the RNA becomes arranged in a conformation in which the charge can be dissipated by sharing it with a partially positive atom such as the scissile phosphorus. This is of course equivalent to saying that as a covalent bond begins to form between the phosphorus and the 2′-oxygen, the burden of the negative charge becomes relieved. If the bond-breaking step of the reaction is slowed by the kinetic bottleneck, the conformation that is observed by X-ray crystallography will partially resemble the transition state, but the excess negative charge distribution will be more localized upon the 2′-oxygen if a covalent bond can only form partially. The excess negative charge residing on the 2′-oxygen will be less unstable at a higher pH than at a lower pH, since the lower pH environment will lead to protonation and thus to relaxation back to the initial-state structure to a greater extent than the higher-pH environment. If this is the case, deprotonation of the 2′-hydroxyl at the active site will be the driving force behind the observed conformational change, and the conformational change, in addition to the actual cleavage reaction, will be seen to be pH dependent. This particular hypothesis postulates that the conformational intermediate structure observed by X-ray crystallography at pH 8·5 is one in which the cleavage-site 2′-hydroxyl is deprotonated. This assertion must, however, be tested

experimentally before it can be regarded as anything but speculative, but it does at least have the merit of some explanatory power.

5.9 Isolating the structure of the cleavage product

The principle of microscopic reversibility (Levine & Bernstein, 1987) states that the mechanisms for forward and back reactions in a simple reaction equilibrium must be identical. Therefore, the catalytic mechanism of the hammerhead ribozyme cleavage and ligation reactions must be the same, meaning that the sequence of events for the reverse reaction should be indistinguishable from those of the forward reaction when time is reversed. Because of this, the structure of the enzyme–product complex that forms just subsequent to cleavage of the ribozyme should be as relevant to the catalytic mechanism as is the structure of the conformational intermediate that forms just prior to cleavage. The Hammond postulate (Hammond, 1955) states that for endothermic reactions, such as the hammerhead cleavage reaction (Hertel & Uhlenbeck, 1995), the transition-state structure will be more product-like than reactant-like. For these reasons, as well as for a complete structural characterization of the hammerhead ribozyme reaction pathway, the structure of the hammerhead ribozyme enzyme–product complex is of particular importance.

Although the self-cleavage reaction proceeds in crystals of the hammerhead ribozyme, allowing trapping and observation of two conformational intermediates that occur prior to catalysis (Scott *et al*. 1996; Murray *et al*. 1998a), the structure of the enzyme–product complex that forms subsequent to cleavage but prior to dissociation has evaded observation until very recently. Upon cleavage, hammerhead ribozyme crystals become highly mosaic, making collection of useful diffraction data on the enzyme–product complex impossible (Scott *et al*. 1996). To solve this problem, a reinforced version of the crystal lattice was used to trap the hammerhead enzyme–product complex, allowing determination of its structure with the aid of a real-space electron density refinement procedure referred to as X-ray holographic reconstruction (Szöke, 1993; Maalouf *et al*. 1993; Somoza *et al*. 1995, 1997; Szöke *et al*. 1997; Szöke, 1998). To circumvent the problem of crystalline disorder caused by RNA cleavage in the crystal, a fraction of modified (or inhibited) RNA substrate (Murray *et al*. 1998a; Scott *et al*. 1995) was mixed with unmodified RNA (Scott *et al*. 1996) during crystallization. Crystals were then grown in which a known (and presumably randomly distributed) subset of the substrate molecules in the crystal are uncleavable or very slowly reactive, conferring sufficient rigidity upon the crystal lattice to allow cleavage of the active RNA substrate in a standard single-turnover reaction under normal conditions without disrupting the diffraction properties of the crystal. In other words, the crystal lattice of the enzyme–product complex was strengthened by randomly incorporating unreactive enzyme–substrate complex reinforcements. The crystal lattice itself was thus used to trap the enzyme–product complex before it becomes disordered (Murray *et al*. 2000).

The X-ray holographic procedure has enabled detection of a significant conformational rearrangement of the cleavage site base in the cleaved hammerhead RNA structure relative to the initial-state structure, as is illustrated in Fig. 7. Instead of being positioned in the catalytic pocket as it is before cleavage, C-17 (the nucleotide 5′ to the scissile phosphate) moves dramatically in such a way as to be almost perpendicular to the Watson–Crick faces of G-5 and A-6 in the catalytic pocket. Several interactions between C-17 and these enzyme-strand residues potentially exist. Most notable are the potential hydrogen bond that forms

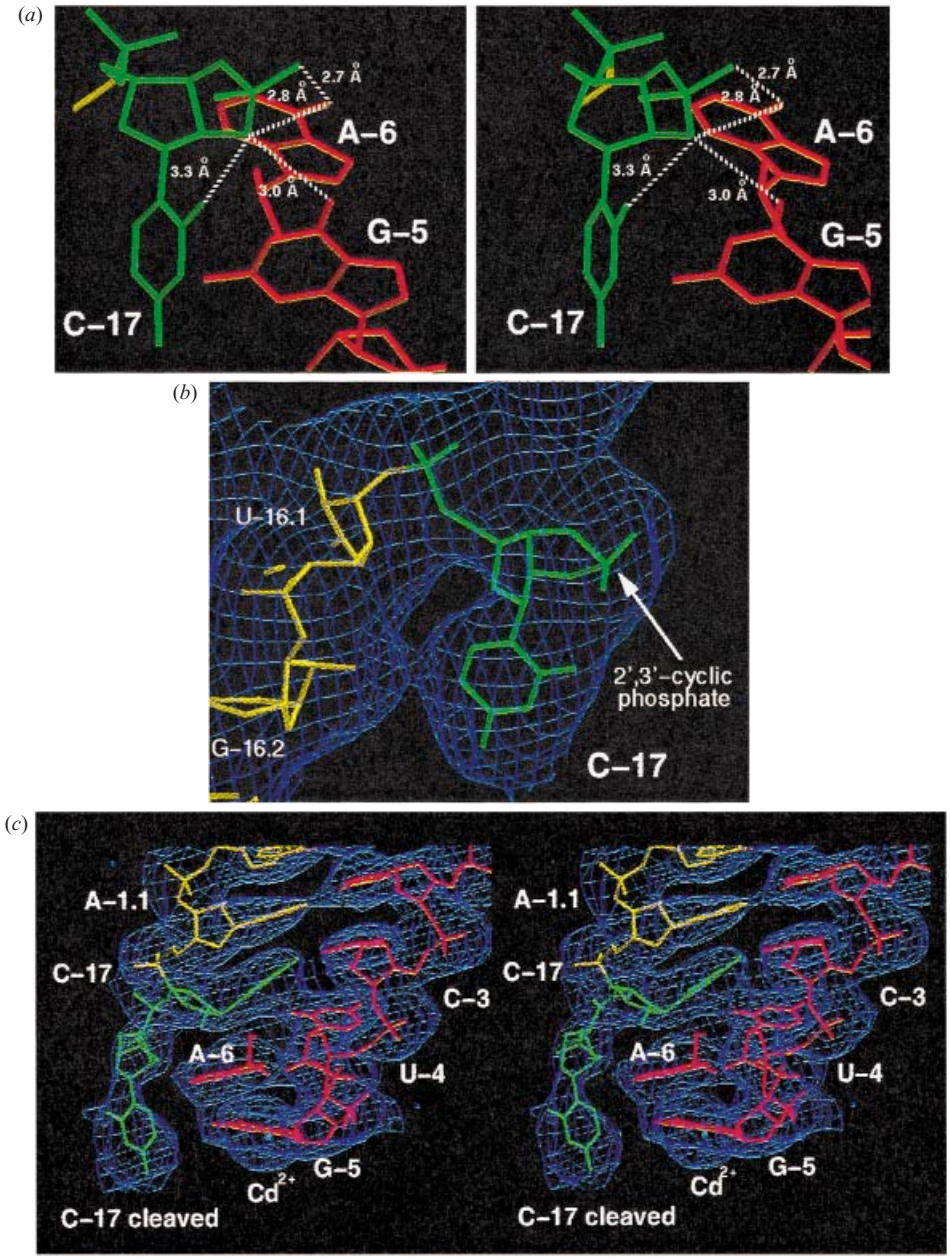


Fig. 7. (*a*) Stereo image of the 2′,3′-cyclic phosphate terminus of the ribozyme substrate complex, showing various distances to the two closest residues of the enzyme strand, G-5 and A-6. Not all distances represent hydrogen bonds, as discussed in the text. (*b*) The EDEN holographic reconstruction of the electron density of the hammerhead ribozyme cleavage product at 60% occupancy, showing density that accommodates the 2′,3′-cyclic phosphate terminus at C-17. The nucleotide and cyclic phosphate were omitted during map calculation. The additional density corresponds to the position of C-17 in the uncleaved substrate. (The holographic reconstruction procedure relies upon 'apodization' or smearing of the data, which yields a map having more rounded features and thus gives the impression of being somewhat lower in resolution compared to the map in (*c*). (*c*) Stereo view of a standard 2Fo-Fc map, calculated in XPLOR 3.8, shows reasonable density for the omitted, 40% occupied C-17 and its cyclic phosphate (shown in green). The density for the remainder of the catalytic pocket region is shown, including that for the uncleaved C-17 (also shown in green).

between the exocylic amine (N6) of A-6 and a cyclic phosphate nonbridging oxygen and the close approach of the keto oxygen (O6) of G-5 to the 2′-oxygen of C-17. These interactions are particularly interesting because they involve functional groups that reside on two residues (A-6 and G-5) that are quite critical for catalysis but whose importance cannot be explained by either the initial-state or intermediate structures (McKay, 1996). Although the potential hydrogen bond forming between the cyclic phosphate of the product and the exocyclic amine of A-6 appears reasonable, neither the keto oxygen of guanine nor the 2′-oxygen of the cyclic phosphate are normally protonated; hence there can be no hydrogen bond between them under ordinary circumstances. C-17 instead appears to form a perpendicular stabilizing aromatic interaction with G-5 that is reminiscent of what is often found in protein structures (Burley & Petsko, 1985). To form a hydrogen bond, either the 2′-oxygen would have to be protonated, or G-5 would need to exist as the enol tautomeric form. Both are unlikely for a stable enzyme–product complex under near-neutral pH conditions, but may have some catalytic relevance.

At least two such potential transition-state interactions should be considered, based upon the structure of the enzyme–product hammerhead ribozyme complex. First, the exocyclic amine of A-6 may hydrogen bond to one of the nonbridging oxygens of the pentacoordinated oxyphosphorane transition state, helping to dissipate the accumulating negative charge. Evidence for a protonated, triester-like transition-state structure has recently been presented (Zhou *et al*. 1998). Second, the potential interaction between G-5 and the 2′-oxygen of C-17 may also be of catalytic relevance if it persists in the transition state. The 2′-oxygen of C-17 in the substrate is initially protonated. If the 2′-H is transferred to O6 of G-5, the cleavage reaction might be initiated as G-5 transiently accepts a proton. Because the enol-like state of G-5 is unfavorable, this proton, or an N1 or N2 proton, would likely be surrendered to the solvent rather quickly, allowing restoration of the uncharged, keto state of G-5. The principle of microscopic reversibility can then be invoked to explain how the relatively rare back reaction is catalyzed: in addition to stabilization of the cyclic phosphate by the hydrogen bond to the exocyclic amine of A-6, the relatively rare keto–enol tautomerization of G-5 can potentially supply a proton to the 2′-oxygen in the (entropically unfavorable) event of nucleophilic attack by the 5′-terminal oxygen of residue 1·1 in the other product strand. Alternatively, the exocyclic amine may instead, or in addition, participate in transition-state interactions that aid proton transfer in both the forward and back reactions in a form of anchimeric assistance. Although these mechanistic proposals have the merit of explaining the requirements for the G-5 and A-6 exocyclic functional groups, they do not address how the 5′-oxygen is stabilized during cleavage, or how it becomes deprotonated when the ligation reaction is catalyzed.

In any case, we now have structures of the hammerhead ribozyme at several points on the self-cleavage reaction pathway. These include the initial-state hammerhead structures, with both active and noncleaving or slowly cleaving substrates, all of which are essentially identical, as well as two freeze-trapped intermediate conformations that form prior to catalysis, and now the structure of the enzyme–product complex. Choosing (1) the initial-state structure, 92) the later conformational intermediate structure, and (3) the ribozyme–product complex structure, we can represent the rather dramatic reaction conformational dynamics with three superimposed structures as shown in Fig. 8.

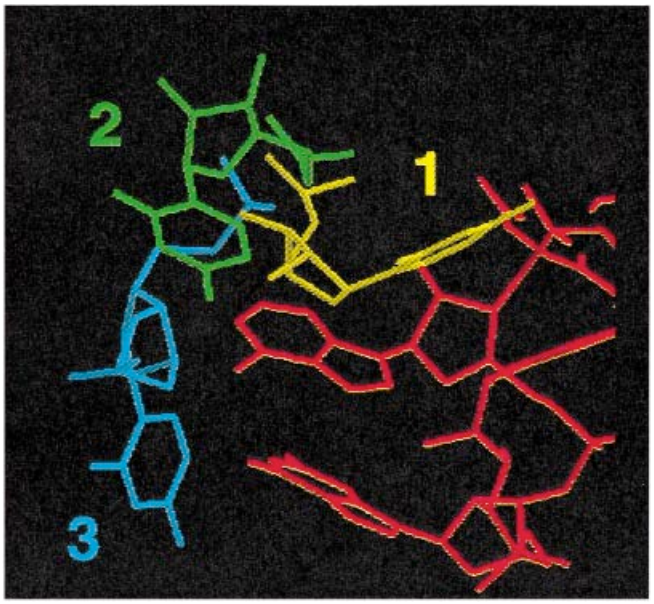


Fig. 8. The progress of the cleavage reaction. The enzyme strand is shown in red, and C-17 is shown in three different positions: (1) the initial-state structure in yellow, (2) the later conformational intermediate structure in green, and (3) the ribozyme–product complex structure in light blue. This gives an impression of the rather dramatic conformational dynamics that the ribozyme undergoes during catalysis.

5.10 Evidence for and against additional large-scale conformational changes

The fact that the cleavage reaction of the hammerhead ribozyme can take place within the confines of the crystal lattice of the initial-state or ground-state structure together with the facts that the cleavage rate in the crystal is on the order of what one would expect in solution (and is actually faster for the particular sequence crystallized) and that the extent of cleavage is actually greater in the crystal than in solution, permit the suggestion to be made that any conformational change that the hammerhead ribozyme must undergo to reach the active structure capable of forming the chemical transition-state must be one that does not involve a global change of the fold in the RNA. This conclusion is corroborated by a number of biochemical studies, including a set of chemical crosslinking experiments that demonstrate that the hammerhead ribozyme initial-state structure as observed in the crystal is at the very least compatible with a rigid distance and orientational constraint imposed upon the Stem I and Stem II helices relative to one another (Sigurdsson *et al*. 1995), numerous FRET analyses (Bassi *et al*. 1996, 1997, 1999), and kinetics experiments undertaken using circular substrates for a ribozyme in the I/II format (Stage-Zimmermann & Uhlenbeck, 1998).

However, a number of other biochemical studies appear to contradict the crystal structure. As noted above in Section 4, these include a number of base-modification experiments that show various conserved residues, especially G-5, G-8 and G-12 in the catalytic core, to be extremely sensitive to modification of their exocyclic functional groups. Although modifications of any of the exocyclic functional groups in each of these three nucleotides have a dramatic effect upon catalysis, there is little effect upon substrate binding and no obvious explanation, in most cases, in terms of the hydrogen bonding pattern observed in the initial-state or intermediate crystal structures. These observations are summarized in McKay (1996).

In addition, modification of several phosphates, including the one observed to bind a divalent metal ion in the original crystal structure (Pley *et al*. 1994), results in a dramatic decrease in catalytic rate. Based upon these results, it has been proposed that the hammerhead ribozyme rearranges from the initial-state structure observed by X-ray diffraction (Fig. 1(*c*); Pley *et al*. 1994; Scott *et al*. 1995) to a significantly different structure that is catalytically active (Peracchi *et al*. 1997, 1998; Wang *et al*. 1999). In this proposed structure, two phosphates that are approximately 20 Å apart in the crystal structure join together to form a single metal binding pocket. One of these phosphates is that of A-9 and is observed to bind a divalent metal ion in the crystal structure (Pley *et al*. 1994; Scott *et al*. 1996) through direct coordination with the nonbridging *pro*-R oxygen (Fig. 9(*a*)). The other, the scissile phosphate, is also said to coordinate the same divalent metal ion directly through its *pro*-R oxygen. The strongest evidence for this mode of metal binding was obtained from simultaneous phosphorothioate substitutions at each of the phosphate *pro*-R oxygens (Wang *et al*. 1999). The deleterious effect of these substitutions upon the Mg^{2+}-catalyzed hammerhead ribozyme cleavage reaction are ameliorated by including a thiophilic metal ion, such as Cd^{2+}, in the reaction mixture.

Each of the two individual phosphorothioate substitutions can be 'rescued' such that the cleavage rate of the modified ribozyme exceeds that of the wild-type ribozyme in reaction mixtures containing both Mg^{2+} and Cd^{2+}. When both the A-9 and scissile phosphates are simultaneously replaced with the phosphorothioates, the doubly modified RNA can again be rescued with reaction mixtures containing both Mg^{2+} and Cd^{2+}, albeit to 1 % of the wild-type activity (Wang *et al*. 1999). This observation has been offered as strong evidence for these two phosphates forming a single metal-ion binding site that assembles upon a proposed transition of the hammerhead ribozyme to a catalytically competent structure from the one observed in the crystal (the so-called ground state). These experiments also lead to the suggestion that the relevant metal ion is bound by the A-9 phosphate and N7 of the adjacent nucleotide, G-10·1, in the ground state (as is observed in the crystal structure) and that the scissile phosphate also becomes coordinated to this metal ion when the conformation changes to that of the catalytically active structure, leading to the formation of a transition state in which the N7 of G-10·1 and both phosphates, via their *pro*-R oxygens, are directly coordinated to the divalent metal ion (Wang *et al*. 1999). In other words (Wang *et al*. 1999), the Stem II helix and those nucleotides augmenting it (Domain II) bind the metal ion and remain unchanged. In the transition to the active structure, the Stem I helix, the cleavage-site residue (C-17) and the conserved bases that surround it (the uridine turn or Domain I) must therefore change conformation (from the ground-state crystal structure) relative to the unchanged part of the molecule in such a way as to enable binding of the scissile phosphate to the same metal ion. The scissile phosphate, according to this scheme, must travel approximately 20 Å from the position that it occupies in the crystal structure.

By constructing a family of model structures that are simultaneously compatible with the octahedral coordination geometry of the bridging divalent cation, the hammerhead ribozyme Stem II and Domain II structure determined by X-ray crystallography, the known requirement for an in-line attack mechanism, and the stereochemical constraints that are inherent to the RNA molecule, it was found that the set of possible structures simultaneously satisfying all of these criteria (i.e., only those stipulated in Wang *et al*. 1999) is quite restricted (Fig. 9*b*). (Murray & Scott, 2000). A number of previous experiments performed upon the hammerhead ribozyme are appropriate to consider in evaluating whether such a

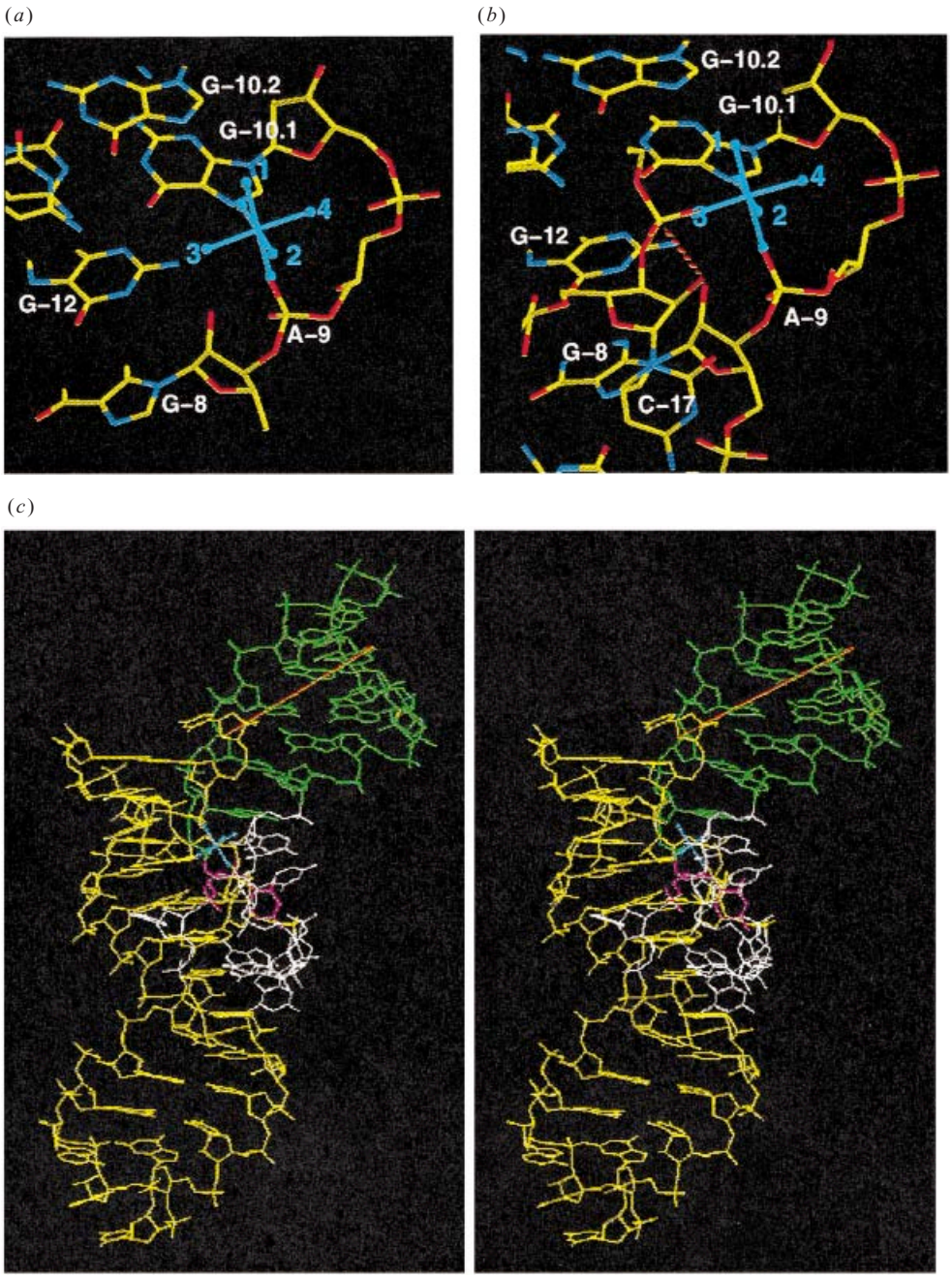


Fig. 9. (*a*) The A-9 phosphate metal binding site as it appears in the crystal structure of the hammerhead ribozyme in the presence of Mn^{2+}. (*b*) The A-9 phosphate metal binding site as it appears in the model of the hammerhead ribozyme when the same metal also coordinates the scissile phosphate adjacent to C-17 at ligation site 3 in the octahedral complex. Models in which the scissile phosphate is coordinated at either position 1, 2 or 4 invariably lead to stereochemical clashes and unphysical bond distances. (*c*) Stereo view of the most plausible model structure consistent with the requirements of the double phosphorothioate experiments and their interpretation. The parts of the RNA shown in yellow (Stem II, Domain II and Stem III apart from U-16·1) and green (stem I) were treated as separate rigid bodies allowed to achieve their most favorable orentiations subject to the constraints imposed by the divalent

conformationally changed molecular structure, however plausible *a priori*, is consistent with the known constraints upon the possible structures of the transition state of the molecule. Perhaps the most definitive of these is one in which the hammerhead ribozyme can be reversibly crosslinked using a chemically engineered disulfide linkage between nonessential residues in the Stem I and Stem II helices (Sigurdsson *et al*. 1995). When two 2′-NH_3 modified nucleotides are incorporated into the hammerhead ribozyme, these allow linkage of the 2′-N atoms to moieties (3-aminobenzyl mercaptan derivatives) that in turn can form disulfide crosslinks between the 2′-NH_3 modified nucleotides in the RNA molecule. In the original experiment, this technique was used to test between two model hammerhead structures (one based on FRET measurements (Tuschl *et al*. 1994), and the other based on the crystal structure) in a decisive manner. Two hammerhead ribozymes were synthesized for this experiment. One permitted residue 2·1 in the Stem I helix to be crosslinked to residue 11·2 in Stem II. The 2′-OHs of these nucleotides are about 13 Å apart in the FRET model, but are 33 Å apart in the crystal structure. The other permitted residue 2·6 of the Stem I helix to be crosslinked to L2·4, a nucleotide occupying a tetraloop terminal position analogous to residue 11·5 in Stem II. These are 11 Å apart in the crystal structure, but are 32 Å apart in the FRET model. Both ribozymes were active within a factor of two of wild-type activity when the disulfide bond was reduced. Upon oxidation, the hammerhead ribozyme crosslinked in a manner consistent with the crystal structure maintained approximately wild-type activity, whereas the activity of the ribozyme crosslinked in a manner consistent with the FRET structure decreased 300-fold. As a further control a ribozyme with a crosslink between residues 2·1 and 10·4 in Stem II showed a similar decrease in activity, despite again having near wild-type activity when the disulfide bond is reduced. These experiments revealed unambiguously that the distance between the 2′-OH of residue 2·6 and that of residue 11·5 (or its equivalent) is less than, or at most equal to, the fully extended length of the crosslink, 16 Å, and that the space between these residues must be unoccupied for the crosslink to form. This covalent distance constraint is compatible with the 11 Å distance observed in the crystal structure and incompatible with the FRET-based model structure.

The disulfide crosslinking experiment does not prove that the crystal structure of the hammerhead ribozyme is the catalytically active structure. It merely proves that in the catalytically active structure, there exists a rigid distance constraint that is consistent with what is observed in the crystal structure. If the crystal structure rearranges to form another structure that is catalytically active, this structure too must have the distance between the 2′-OH of residue 2·6 and that of residue 11·5 (or its equivalent) less than or at most equal to 16 Å. In the model of the proposed transition-state structure, the distance between the 2′-oxygen of residue 2 ± 6 and that of 11·5 (assuming Stems I and II continue as standard A-form RNA helices) cannot be forced to become less than 21 Å. Moreover, a line passing through these two 2′-oxygen atoms passes through the Stem helical axis diagonally, meaning that even if

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metal ion coordination geometry (blue) and the requirement for maintaining the connectivity of the phosphate backbone. The residues allowed to vary in position to achieve this connectivity are shown in white, and the cleavage site base is shown in pink. The distance required to be spanned by a chemical crosslink less than or equal to 16 Å in the catalytically active molecule is shown in red. The actual distance in the model cannot be forced to be less than 20 Å without unwinding the helices. Moreover, the Stem I helix would be forced to unwind completely in order to prevent a steric clash with the crosslinking moieties.

the covalent crosslink were stretched to 21 Å, it would also be required to pass through the RNA helix, an obvious impossibility (Fig. 9(*c*)). The only way in which the model structure can be forced to accommodate the crosslink without steric clashes and without stretching beyond 16 Å is for the Stem I helix to fully unwind. Therefore it is reasonably safe to conclude that all of the physically plausible model structures that are consistent with Wang *et al*. (1999) can be eliminated based upon the disulfide crosslink-imposed distance constraints.

The model structure therefore represents a concrete, testable hypothesis of the claim that a single metal ion binds both the A-9 and scissile phosphates in the active hammerhead ribozyme. It was found that the currently existing disulfide crosslinking data, as well as several other sets of experimental results, conflict even with the most plausible structures that are based upon the observations and proposals in the Herschlag analysis. It can therefore be suggested that a hammerhead ribozyme catalytic mechanism in which the A-9 and scissile phosphates are bridged by a single divalent metal ion that coordinates both phosphates is structurally unsound.

Seen in this light, several experimental observations described above now have fairly straightforward interpretations. For example, the previous observation that divalent metal ions are not strictly required for hammerhead ribozyme catalysis (Murray *et al*. 1998b) presents some difficulties for the proposed conformational change mechanism. This lack of a divalent metal ion requirement would be hard to reconcile with a requirement for a metal ion that bridges the A-9 and scissile phosphates for catalysis. The extremely limited rescue (to 1 % of wild-type activity) of the hammerhead ribozyme containing phosphorothioates at both the A-9 and scissile phosphates (Wang *et al*. 1999) can be easily explained if the metal is in fact stabilizing a catalytically *inactive* conformation (with the residual activity accounted for in terms of acknowledged sample impurity). In addition, the lack of a thio-effect in hammerhead ribozymes in which the scissile phosphate has both the *pro*-R and the *pro*-S oxygens simultaneously replaced with sulfur atoms as described in Section 3.2 (W. B. Derrick, C. Greef, M. Caruthers & O. C. Uhlenbeck, unpublished results), become understandable if there is no requirement for a divalent metal ion to bind to the scissile phosphate. Finally, the acceleration in the rate of cleavage and enhancement of the extent of cleavage of a hammerhead ribozyme sequence upon crystallization (Murray *et al*. 1998a) becomes more understandable if a large-scale conformational rearrangement that would require disruption of the crystal lattice is not required for catalysis.

5.11 NMR spectroscopic studies of the hammerhead ribozyme

Although a complete three-dimensional structural determination of the hammerhead ribozyme by NMR spectroscopy has to date not been possible, considerable amounts of information about the structure and dynamics of this ribozyme have been obtained. Although the results in general are consistent with what has been observed by X-ray crystallography, there are some important differences, and also much complementary information. The predicted canonical secondary structure (Fig. 1(*a*)) was first verified by NMR (Caviani-Pease & Wemmer, 1990); this study also revealed the absence of resonances due to tertiary base-pairs in the low-field spectrum, both in the absence and presence of Mg^{2+}. These studies were carried out on a hammerhead RNA subsequent to cleavage, so it was unclear whether the lack of additional resonances corresponding to tertiary structural interactions was a consequence of possible unfolding subsequent to cleavage or was relevant to the uncleaved structure as

well. Additional experiments on hammerhead ribozymes in which the RNA substrate was replaced with an uncleavable RNA analogue also verified the canonical secondary structure but did not reveal NOEs that could be shown to correspond to tertiary base interactions (Heus & Pardi, 1991).

Although *de novo* solution of the hammerhead ribozyme appears to have been an intractable problem, knowledge of the crystal structure enabled the extraction of more useful information from the NMR data. By comparing the NMR spectra of cleaved and uncleaved cleavage RNAs, it was shown that the three canonical helices surrounding the conserved catalytic core were present both before and after cleavage, as were the sheared G–A base-pairs in the conserved core. The tandem GA pairs were also observed to form even in the absence of Mg^{2+}, but a significant structural change of the conserved core of the hammerhead ribozyme–substrate complex was observed to take place upon cleavage of the substrate. Specifically, an NOE between I-4 of the uridine turn, and U-7, the nonconserved nucleotide in the augmented Stem II helix, indicative of a U–U base-pair, was observed in the cleaved, but not the uncleaved, hammerhead RNA structure. This interaction entails a significant rearrangement of the catalytic core region subsequent to (or perhaps even during) catalysis (Simorre *et al*. 1997). This observation is not inconsistent with what is observed in the various crystal structures if the rearrangement takes place subsequent to cleavage; in the case of the cleaved RNA crystal structure, it is likely that the crystal lattice prevents the RNA from relaxing to the structure observed in solution by NMR. However, if the rearrangement takes place prior to catalysis, this would be inconsistent with what has been observed in the crystal structure but would perhaps be consistent with the larger-scale conformational changes discussed in the previous section.

The effect of the cleavage site nucleotide's identity on the solution structure of the hammerhead ribozyme has also been investigated by NMR. Unlike most of the core residues, the identity of the cleavage site base is relatively unrestricted; it can be anything but G. As predicted, a G at the cleavage site was observed by NMR to form a canonical base-pair with C-3 of the uridine turn, thus presumably destroying the ability of the hammerhead RNA to form the tertiary structure required for catalysis. Substitution of U for C had little structural effect, but an A at the cleavage site revealed a rather different mode of binding (Simorre *et al*. 1998). The crystal structure of the hammerhead ribozyme with A at the cleavage site, on the other hand, reveals a structure almost identical to that of the original structures with C at the cleavage site (Beigelman & Scott, unpublished results). This suggests that the dominant conformation in solution is that which is seen by NMR, and the crystal lattice induces formation of the other conformation selectively during crystallization.

Finally, ^{31}P NMR has recently been used to identify a novel high-affinity metal-binding site in the hammerhead ribozyme that was not previously identified in any of the crystal structures. This metal binding site involves the phosphate at A-13, approximately twofold symmetric to the A-9 metal site observed in the crystal structure for the approximately twofold symmetric tandem G–A pairs (Hansen *et al*. 1999). An apparent dissociation constant for Mg^{2+} of 250–570 μM was observed for this site. Since this site has not been observed to be occupied in the crystal structure, and since the phosphate of A-13 is in a rather different conformation from that at A-9 (the latter is essentially 'in-line'), this result indicates that some differences between the crystal structure and the solution structure (or at least between their potential flexibilities) are likely to exist.

6. Concluding remarks

Soon after the discovery of catalytic RNA, the hammerhead ribozyme became the rather intense focus of experimental activity because, as a molecule about an order of magnitude smaller than the Group I intron and RNase P RNA, it appeared to be the most experimentally tractable. Its small size and simple cleavage reaction chemistry held the implicit promise that it would be the catalytic RNA most likely to reveal some of the fundamental principles of RNA catalysis. This was perhaps optimistic, for even with the rather formidable array of experimental techniques brought to bear on the problem of hammerhead ribozyme catalysis (synthetic nucleotide biochemistry, phosphorothioate substitutions, chemical crosslinking, metal ion studies, molecular modeling studies, conventional and time-resolved X-ray crystallography, multidimensional heteronuclear and ^{31}P NMR spectroscopy, fluorescence resonance energy transfer, transient electric birefringence and mutational analyses), a somewhat discordant picture of how the hammerhead ribozyme works has emerged. Broadly speaking, the results of the physical techniques applied to the problem of hammerhead ribozyme catalysis tend to permit their practitioners to suggest that relatively localized conformational changes occur in the course of the cleavage reaction, whereas the results of at least some of the biochemical experiments (notably excluding the chemical crosslinking experiments) have been interpreted to indicate the presence of a relatively large-scale conformational change that takes place in terms of catalysis. This is in a way unfortunate, because it means that many years of intensive study haven't yielded a definitive answer about how even this most simple of RNA enzymes works. From the pessimists's point of view, if one extrapolates to the larger catalytic RNA molecules, the situation would seem completely hopeless.

However, it is in retrospect possible that the assumption that the smallest and mechanistically simplest ribozyme would prove to be the most experimentally tractable might itself by flawed. Perhaps the hammerhead RNA, a relatively small molecule consisting of a minimal number of conserved nucleotides, might in fact be more difficult to study because it has little if any built-in structural redundancy, making interpretation of even the most minimal perturbations difficult. A large ribozyme such as the Group I intron may simply be more structurally stable and thus more robust with respect to the structural perturbations induced by natural and unnatural nucleotide substitutions. The division between 'enzyme' and 'substrate' in the hammerhead ribozyme system is essentially arbitrary, whereas with RNase P, it is completely natural. The extreme structural simplicity of the hammerhead RNA and its unnatural division into enzyme and substrate components may then be responsible, at least in part, for our inability to reconcile some of the biochemical experimental results with the more physically oriented experiments; the hammerhead ribozyme in other words might push the limits of validity for interpreting the results of enzyme kinetics and mutagenesis experiments within conventional frameworks, whereas the large ribozymes may well be better behaved in this respect.

My personal bias is to trust the interpretation of the results of the physical techniques over those of the biochemical techniques because the results of the former tend to be less dependent upon interpretations that are based on a set of assumptions whose validity has not been established unambiguously. Does, for example, a phosphorothioate substitution that shows a pronounced but rescuable thio-effect necessarily entail a metal ion binding site at this location in the unmodified RNA? The assumption has of course been that it does, but the lack

of a strict requirement for divalent metal ions in hammerhead ribozyme catalysis shows that there must be some other explanation. Ultimately, we would hope to be able to resolve, or at least explain, the differences between the two sets of results. Perhaps it is only in doing this that the true insight into how RNA catalysis can take place will emerge.

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