REVIEW

nature structural & molecular biology

Ribozyme catalysis: not different, just worse

Jennifer A Doudna[1] & Jon R Lorsch[2]

Evolution has resoundingly favored protein enzymes over RNA-based catalysts, yet ribozymes occupy important niches in modern cell biology that include the starring role in catalysis of protein synthesis on the ribosome. Recent results from structural and biochemical studies show that natural ribozymes use an impressive range of catalytic mechanisms, beyond metalloenzyme chemistry and analogous to more chemically diverse protein enzymes. These findings make it increasingly possible to compare details of RNA- and protein-based catalysis.

With apologies to Jeremy Knowles and his seminal review, "Enzyme catalysis: not different, just better".

(*Nature* **350**, 121–124, 1991).

The emergence of efficient and highly specific catalysts for biochemical reactions was key to the evolution of living systems. Although protein enzymes dominate modern cell biology, discoveries of catalytic RNA molecules, called ribozymes, fueled the suspicion that nucleic acids were key to the origin of biocatalysts, in part because RNA plays central roles in the fundamental process of protein biosynthesis in all cells. According to the 'RNA world' hypothesis, RNA once served as both the genetic material and the principal biocatalyst in living systems. As this primitive RNA-based metabolism evolved, requirements for more sophisticated enzymes with superior catalytic powers are thought to have stimulated the transition to protein-mediated catalysis. It seems possible that naturally occurring ribozymes present in organisms ranging from bacteria to humans are in fact remnants from this envisioned RNA-dominated era. If this is true, at least some of the catalytic functions of modern enzymes were originally carried out by ribozymes.

A decade's worth of *in vitro* selection and evolution experiments have proven that ribozymes are indeed capable of catalyzing a broad range of chemical reactions and can provide rate enhancements respectable enough, perhaps, to have sustained basic life forms on the early Earth. As researchers have studied both naturally occurring and *in vitro*–evolved ribozymes, an underlying question has persisted: is ribozyme catalysis the same as or different than protein catalysis? On the face of it this question seems reasonable; proteins and nucleic acids are very different macromolecules, after all. Proteins have a variety of functional groups available for use in catalyzing reactions and in folding into stable, complex structures. In contrast, ribozymes have only four bases, all of which look more or less the same, and although they can fold into complex shapes, it is not clear that nature can fine-tune their structures with quite the same precision as the diverse array of amino acid side chains allows. But is the question really reasonable? At a chemical level, how different could RNA-based catalysis be from protein-based catalysis?

If one strips away the macromolecule from the catalyzed reaction, there are only a limited number of mechanisms through which a reaction can be catalyzed. The overall goal is to stabilize the transition state of the reaction relative to the ground state. To do this, the catalyst can pay for the cost of entropy lost in achieving the transition state by positioning the substrates in the appropriate configuration[1–3]. The catalyst can also make stronger interactions with the transition state than with the ground state, thus lowering the enthalpic difference between the two states. Acidic and basic groups can participate in lowering the transition state energy by stabilizing developing charges[4]. Acids and bases can also be used to provide necessary pathways for protons to move into and out of the active site during the course of the reaction[5]. In many reactions untenably large energy barriers would occur if facile pathways for proton movement from one atom to another did not exist[1] and thus creating these pathways is necessary for a macromolecule to be able to get on with the business of stabilizing the transition state of the reaction relative to the ground state. If the ground state of the reaction of interest is the enzyme–substrate complex, the reaction can be accelerated by decreasing the stability of this state without a corresponding decrease in transition state stability. A catalyst can also break the reaction pathway into new steps by covalently altering the substrate, thereby chopping one barrier into several smaller ones and possibly making the rate-limiting transition state(s) easier to stabilize.

The mechanisms described above are general and make no assumptions about the nature of the molecule catalyzing the reaction. Although it is not necessary that a certain class of macromolecular catalysts use all of the above strategies, it is certain that these catalysts will use a subset of them, and it seems very unlikely (we would like to submit unimaginable) that they will invent any new ones. Thus once again with ribozymes, as with so much else, the Ecclesiastes principle holds: "There is nothing new under the sun." So far, the best studied classes of ribozyme-catalyzed reactions—self-cleaving ribozymes, self-splicing introns and the ribosome—demonstrate this principle. This review highlights new insights into ribozyme catalysis as well as some of the many remaining challenges to understanding detailed mechanisms of action of RNA enzymes.

[1]Department of Molecular and Cell Biology, Department of Chemistry, Howard Hughes Medical Institute, University of California at Berkeley, Berkeley, California 94720, USA. [2]Department of Biophysics and Biophysical Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, USA. Correspondence should be addressed to J.A.D. (doudna@berkeley.edu) and to J.R.L. (jlorsch@jhmi.edu).

Published online 3 May 2005; doi:10.1038/nsmb932

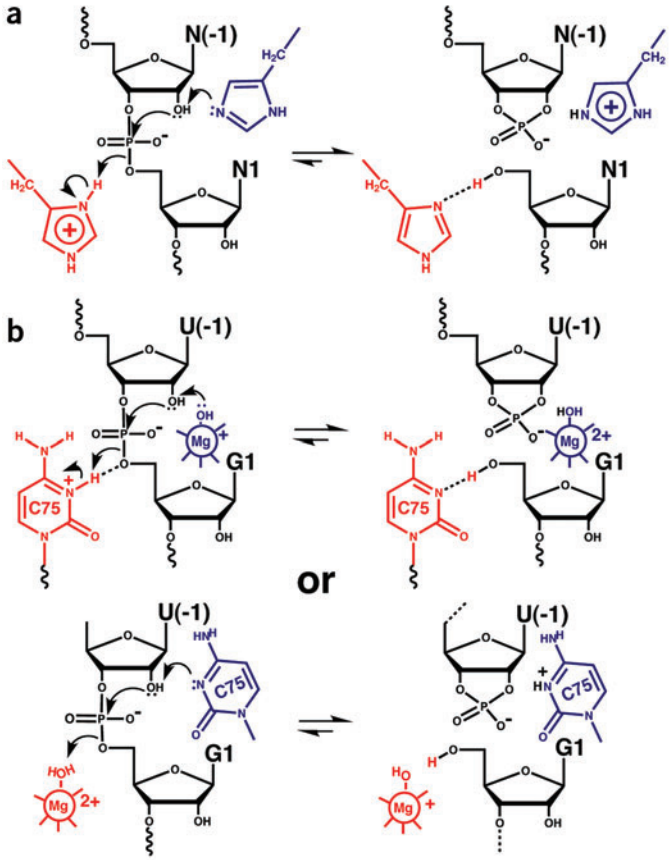

Figure 1 Comparison of proposed mechanisms for RNA strand scission catalyzed by the protein enzyme RNase A and the HDV ribozyme. In each case, the reaction involves nucleophilic attack of the 2′ hydroxyl on the adjacent phosphorous atom and produces products with 2′,3′-cyclic phosphate and 5′-hydroxyl termini. In this and subsequent figures, general bases are blue and general acids are red. (**a**) Mechanism of catalysis by RNase A, in which two histidine side chains function as general base and general acid, respectively. (**b**) Two proposed mechanisms for HDV ribozyme–mediated cleavage: top, C75 acts as a general acid; bottom, C75 acts as a general base. Not all known or proposed interactions are shown; squiggly lines represent connections to the rest of the RNA chain. (**c**) Precursor and product states of the ribozyme determined by X-ray crystallography[31,48]; note that in the precursor, the 5′-oxygen leaving group coordinates to a hydrated magnesium ion, whereas in the product state, it is within hydrogen bonding distance of the N3 of C75. **Figure 1c** was prepared by A. Ke using VMD (http://www.ks.uiuc.edu/Research/vmd/).

RNA strand scission

Nature has produced an abundance of protein enzymes and five known classes of naturally occurring ribozymes that promote phosphodiester cleavage in RNA substrates (**Fig. 1**). Protein enzymes that catalyze nucleophilic attack at a phosphate within RNA or a ribonucleotide use a variety of chemical mechanisms. For example, mammalian adenylyl cyclases function by a two-metal-ion mechanism[6], ribonuclease A uses two histidines for general acid-base catalysis[7] (**Fig. 1a**), and the anthrax adenylyl cyclase exotoxin uses one histidine and a coordinated metal ion to activate the attacking nucleophile and stabilize the leaving group, respectively[8]. Interestingly, structural and mechanistic studies show that self-cleaving ribozymes also catalyze phosphodiester bond scission by a variety of mechanisms, demonstrating a breadth of catalytic potential surprisingly analogous to proteins (**Table 1**). Three of these ribozymes are small, ~40–120-nucleotide RNAs for which multiple crystal structures are available in each case. The hammerhead, hepatitis δ virus (HDV) and hairpin ribozymes all catalyze site-specific self-cleavage resulting in products with 2′,3′-cyclic phosphates and 5′-hydroxyl termini during rolling circle replication of the viral or virusoid RNAs in which they reside. Although these ribozymes promote the same chemical reaction as many protein ribonucleases (**Fig. 1**), they act only at specific phosphodiester bonds by using base-pairing and other interactions to align the cleavage site within the RNA active site. The evolutionary maintenance of these sequences may result in part from the relative ease of evolving efficient and site-specific self-cleaving RNA motifs[9,10].

The hammerhead ribozyme mediates rolling circle replication within circular virus-like RNAs that infect plants. Although the hammerhead can be trimmed to a tiny ~40 nucleotides, natural hammerhead ribozymes include additional nonconserved sequences that stabilize the active conformation at physiological magnesium ion concentrations[11–13]. The simplicity of the minimized hammerhead secondary structure lent itself to the design of two-piece constructs in which the strand containing the cleavage site was separated from the rest of the self-cleaving RNA. By treating one strand as the substrate and the other as the enzyme, multiple-turnover cleavage occurred with a typical rate of 1 molecule per minute *in vitro*, consistent with a 10^6-fold rate enhancement over uncatalyzed nonspecific RNA hydrolysis[14] (for comparison, RNase A accelerates the same reaction $>10^{12}$-fold; ref. 15. The discovery that a catalytic requirement for magnesium was obviated at high (4 M) monovalent salt concentrations[16–18] suggested two distinct possibilities: these ribozymes use a different catalytic mechanism in the presence of high, nonphysiological concentrations of monovalent salts, or the divalent metal ion requirement at low salt concentration serves a structural rather than a chemical function.

Despite an abundance of crystal structures of 'minimal' and catalytically active hammerhead ribozymes[19–25], the positions and functions of bound divalent metal ions have remained elusive. Although several divalent ions were unambiguously identified in these structures, they were not situated close enough to the site of catalysis to support a direct

Table 1 Ribozyme mechanisms

	Crystal structure(s)	Catalytic strategies
Hammerhead	Yes	Substrate orientation; metalloenzyme?
Hairpin	Yes	Electrostatic transition state stabilization; general acid-base?
HDV	Yes	General acid-base; metalloenzyme
Group I introns	Yes	Metalloenzyme; substrate orientation, approximation
Group II introns	Parts	Metalloenzyme
Ribosome	Yes	Substrate orientation, approximation; substrate-assisted catalysis

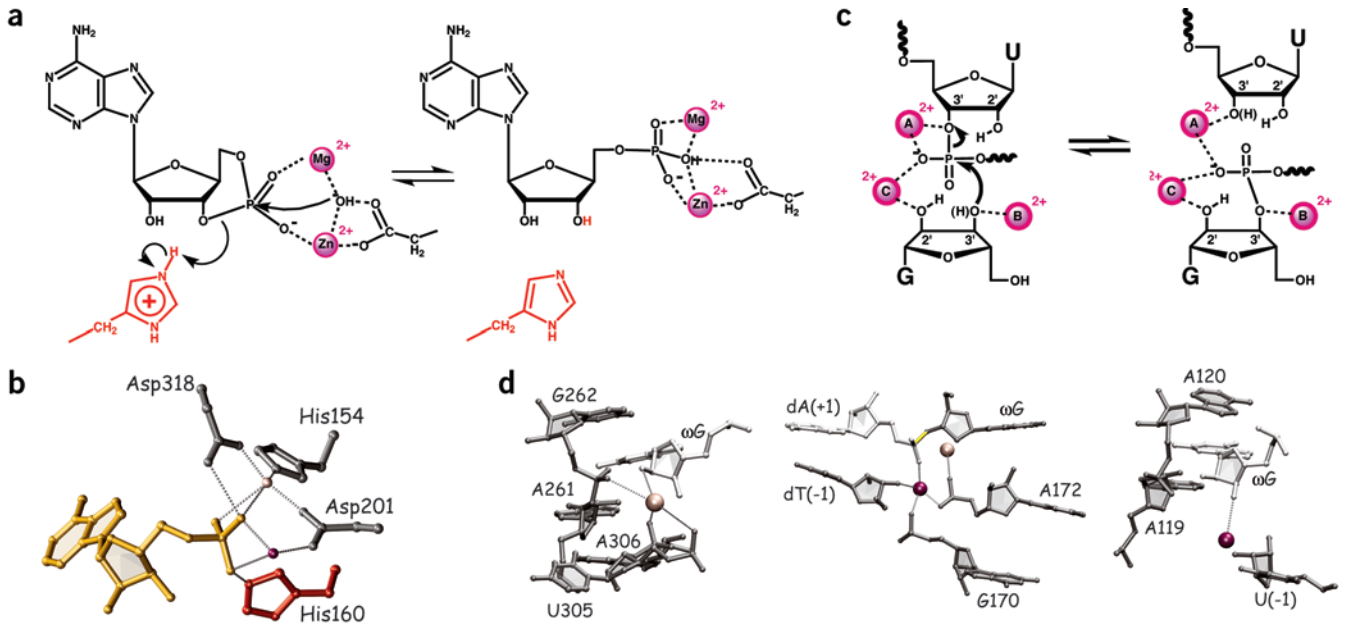

Figure 2 Comparison of proposed mechanisms for hydrolysis of phosphodiesters catalyzed by 3′,5′-cyclic nucleotide phosphodiesterase 4 (PDE4) and the group I intron. (**a**) Two metal ions, a Mg^{2+} and a Zn^{2+}, in the active site of PDE4 (ref. 103) coordinate either a hydroxide ion or a water molecule (or conceivably an oxide[104]) and, along with an aspartate, are thought to orient the OH^- or H_2O and promote its attack on the phosphorus of the cyclic nucleotide. The metals also serve to orient and polarize the cyclic phosphate group. A histidine is proposed to act as a general acid, protonating the leaving group (3′ oxygen). (**b**) PDE4 active site as determined by X-ray crystallography[105], with substrate in orange and oriented as in **a**. (**c**) A mechanism involving three metal ions has been proposed for the group I intron based on extensive sulfur and amino group substitution of active site oxygens[71–73]. Not all known or proposed interactions are shown. (**d**) X-ray crystallographically determined active sites of the (i) *T. thermophila* (3.8 Å resolution), (ii) *Azoarcus* (3.1 Å resolution) and (iii) Twort (3.6 Å resolution) group I introns show the precursor, intermediate (poised to initiate the second step of self-splicing) and post-cleavage states, respectively[79–81]; scissile bond in (ii), yellow; metal ions, purple (A in **c**) and pink (C in **c**); ωG, white (i–iii). Note that the substrate strand in the 3.1 Å *Azoarcus* structure contained all 2′-deoxy residues; in this structure, the metal ion in pink was a potassium ion, whereas a related structure at lower resolution (3.7 Å) with a single 2′-deoxy substitution at the cleavage site contained a magnesium ion at the equivalent position[79]. **Figure 2b,d** was prepared by A. Ke using VMD (http://www.ks.uiuc.edu/Research/vmd/).

role in RNA cleavage. Site-specific substitution of phosphate oxygens with sulfur atoms was used to disrupt divalent metal ion–binding sites potentially involved in catalysis, coupled with attempted 'rescue' of catalysis by these derivatives using thiophilic metal ions[26–28]. Results from these experiments suggested direct simultaneous coordination of a single metal ion by the scissile phosphate and a second phosphate oxygen located 20 Å away in the crystal structure[27,29]. It was proposed that the crystal structures might represent the 'ground state' conformation of the hammerhead ribozyme and that before catalysis the RNA conformation changes substantially but transiently to bring the critical catalytic metal ion proximal to the cleavage site. Molecular modeling and kinetic analysis of the hammerhead cleavage reaction in the presence of monovalent versus divalent salts support the alternative idea that divalent metal ions are not essential to the catalytic step but instead stabilize the active ribozyme structure[17,18,24,30].

The HDV ribozyme and the hairpin ribozyme catalyze the same chemical reaction as that of the hammerhead, and they are likewise responsible for cleaving intermediates generated during rolling circle replication of a human pathogen and a plant virus satellite RNA, respectively. Crystal structures of these ribozymes showed that in each case the RNA forms an enclosed cleft in which strand scission takes place[31,32]. The unexpected possibility that RNA might use general acid-base chemistry, in which nucleobases directly contribute to catalysis by donating or accepting protons during the chemical step of the reaction, was first suggested by the structure of the self-cleaved form of the HDV ribozyme (**Fig. 1b,c**). In this structure, the single most catalytically critical residue, a cytidine (C75), is located in a metal ion–free cleft near the reaction leaving group. Likewise, in an inhibitor-bound form of the hairpin ribozyme, four functionally critical active site purine bases line the active site cavity and one, G8, makes hydrogen bonds to the scissile phosphate[32]. Could these nucleobases play direct roles in ribozyme catalysis? Although protein side chains with near-neutral pK_a values can act as general acids or bases readily *in vivo*[5], the lack of RNA functional groups with pK_a values near physiological pH (6–7) means that for RNA to function in this way, one or more of its functional groups must have a pK_a substantially shifted toward neutral[2] or a group with a nonoptimal pK_a must be used[33]. Substantial pK_a shifts have been documented for A and C residues within small functional RNAs, where the structural environment of the nucleotide favors protonation of a ring nitrogen of the base[34–38].

Proving whether and how general acid-base catalysis might work in the HDV and hairpin ribozymes remains an outstanding challenge. Mutation of C75 slows the rate of HDV ribozyme catalysis by ~10^5-fold (ref. 39), similar to the effect of mutating either of the general acid-base catalytic histidines in ribonuclease A[40]. More modest but substantial 10–350-fold catalytic rate reductions occur in hairpin ribozymes lacking G8, A9 or A10 (ref. 41). In the HDV ribozyme, a network of potential hydrogen bonds to C75 is consistent with stabilization of a protonated form of the base that might allow it to donate or accept a proton at some stage during catalysis[31]. This feature could be very useful for mediating catalysis by pulling a proton off the attacking 2′-oxygen nucleophile, or by providing a proton to the 5′-oxygen leaving group (**Fig. 1b**). But does this in fact occur? Imidazole and

nucleotide analog rescue of ribozymes with mutations at C75 (ref. 42), correlation of reaction pK_a values with those of various imidazole analogs[43], kinetic isotope effects and detailed analysis of metal ion contributions to catalysis[44–46] support a direct role of C75 in proton transfer during catalysis. However, the pK_a of the active site C is not substantially shifted in the ground state[47], and its precise catalytic role remains elusive. More recently, structures of the precursor form of the HDV ribozyme showed that a hydrated magnesium ion binds the active site in the precleaved state, coordinated directly or through a water molecule to the leaving group oxygen[48]. In the precursor active site, C75 is positioned near the 2′-hydroxyl nucleophile, suggesting possible general-base function to initiate the reaction by deprotonating the 2′-hydroxyl nucleophile (**Fig. 1c**).

The situation is murkier still for the hairpin ribozyme. Both single-molecule and ensemble kinetic studies have shown that the rate of the reaction is dependent on pH under conditions where no large scale conformational changes are rate-limiting, and these curves give an apparent pK_a for a group (or groups) in the reaction of ~6.5, consistent with a general acid or base being at work[33,49,50]. In crystal structures of the ribozyme, several purine bases—G8, A9, A10 and A38—are close enough to the site of chemistry to play a role in acid-base catalysis (possibly mediated by a bridging water molecule)[32,51]. Nucleobase rescue experiments have argued against a role for G8 as an acid or base, however[41,49], and pH-dependent nucleotide analog interference experiments cast doubt on the proposed use of A38 as a general acid-base, although this study did suggest that ionization of A10 is important in the mechanism of action of the ribozyme[52]. Regardless of whether a general base or acid is at work, the lack of a requirement for divalent metal ions during hairpin ribozyme cleavage implies that it uses a metal ion–independent mechanism[53,54]. The crystal structure of the ribozyme bound to a transition state analog suggested that the hairpin uses multiple hydrogen bonds to stabilize the transition state and thus catalyze the reaction[51]. It has also been suggested that the pH-dependence of the reaction may reflect the use of a protonated base to electrostatically stabilize developing charge in the transition state, rather than actual proton transfer to the reacting molecule(s)[49,52]. And of course, pH studies being what they are, the possibility that the pH-dependence arises for more byzantine reasons, such as a small, hard-to-detect proton-accelerated conformational change or the composite effects of multiple titrating groups, cannot at this point be excluded.

For at least two of these small ribozymes, the hammerhead and the HDV, it has proved difficult to correlate structural information with all of the information derived from biochemical studies of the mechanisms of catalysis. These discrepancies can be explained if the structures determined for these small ribozymes do not represent the structures that actually stabilize the transition states for the reactions, perhaps because conformational changes must take place during the reaction cycle to attain the active states. A considerable body of evidence supports the notion that the small ribozymes undergo conformational rearrangements during the course of catalysis[25,48,55–58]. For example, Blount *et al.* showed that attaching bulky groups to the 2′ positions of several bases in the hammerhead markedly reduced the rate of the catalyzed reaction, even though the crystal structures indicated that these modifications should be easily accommodated, suggesting that the added groups interfere with a required conformational change[56]. In larger ribozymes such as the group I intron, peripheral domains outside of the catalytic core help to stabilize the active structure of the RNA[59–61]. In fact, it has recently been found that structures in viroid RNAs outside of the hammerhead catalytic domain greatly enhance catalysis by the ribozyme, possibly by stabilizing the active state[11,62]. Determination of the structures of these extended hammerhead ribozymes could prove very enlightening.

RNA splicing

Distinct from RNA strand scission or self-cleavage, splicing involves the excision of an intervening sequence, or intron, from precursor transcripts with concurrent ligation of the flanking sequences, or exons, to form a mature RNA. Introns, perhaps the remnants of an ancient mechanism for increasing the information content and adaptability of RNA, occur widely within precursor transcripts of eukaryotic, and a few viral, messenger RNAs. Splicing of these intervening sequences is catalyzed by the spliceosome, a large and dynamic RNA–protein complex. However, two different classes of autocatalytic introns interrupting genes for rRNA, tRNA and mRNA in protozoan nuclei, fungal mitochondria, algal chloroplasts, bacteria and bacteriophages are capable of self-excision. The group I class, defined by nine base-paired elements (P1–P9), accomplishes splicing by a two-step transesterification mechanism initiated by an exogenous guanosine nucleoside or nucleotide. In a reaction chemically analogous to that catalyzed by phosphodiesterase (**Fig. 2a**,**b**), the 3′ hydroxyl of the bound guanosine substrate attacks the 5′-splice site phosphate and attaches to the 5′ end of the intron (**Fig. 2c**). In a second step, the 3′ OH of the 5′ exon attacks the phosphate at the 3′-splice junction, ligating the exons and excising the intron. In contrast, the group II class of introns share a different, generally larger secondary structure and a distinct splicing mechanism. Here, the 2′ OH of an internal adenosine within the intron serves as the initiating nucleophile, cleaving the 5′-splice site phosphodiester bond and forming a 2′-5′ linkage with the end of the intron. Subsequently, the 3′ OH of the 5′ exon attacks the 3′-splice junction phosphate, ligating the exons and releasing the branched 'lariat' intron. Though much has been made of the mechanistic similarity between group II introns and the spliceosome, it remains unclear whether the two are evolutionarily related and whether the spliceosome is fundamentally a ribozyme. Although models of group II intron architecture[63,64] and molecular structures of limited regions of the intron[65,66] are available, detailed mechanistic insights await further structural and biochemical investigations.

Much more is known about group I intron catalysis, based on both X-ray crystallographic structure determinations and painstaking functional group substitutions and kinetic and thermodynamic analyses. The excised intron retains the active site for transesterification and can thus be redesigned to create a true catalyst that cleaves or ligates exogenous substrate molecules. Using the *Tetrahymena thermophila* version of this RNA enzyme together with oligonucleotide substrates containing various chemical modifications, the reaction pathway has been dissected in detail[67,68]. The ribozyme recognizes a double-stranded RNA substrate containing the 5′-splice site through interactions with specific 2′ hydroxyl groups, positioning it for attack by the 3′ hydroxyl of the bound guanosine cofactor. After splice site cleavage with inversion of stereochemical configuration[69,70]—consistent with an associative S_N2-type reaction—products are released and the ribozyme is ready to bind to a new substrate molecule.

Analogous to protein enzymes that promote phosphoryl transfer reactions, catalysis by group I introns requires divalent metal ions. Considerable effort has thus focused on determining the locations and identities of catalytically important metal ions in the ribozyme active site. By substituting individual phosphate or ribose oxygen atoms with sulfur or with an amino group, and then testing for a metal ion specificity change, three magnesium ions were proposed to contribute directly to catalysis[71–73]. In this model (**Fig. 2c**), one metal ion (A) stabilizes the developing negative charge on the leaving group oxygen in the transition state and also destabilizes the bound substrate in the ground state[74,75]. The second metal (B) helps deprotonate the 3′ oxygen of the G nucleophile, and the third (C) may aid both precise substrate positioning and, along with metal ion A, stabilization of the trigonal bipyramidal transition state[73] (**Fig. 2a**).

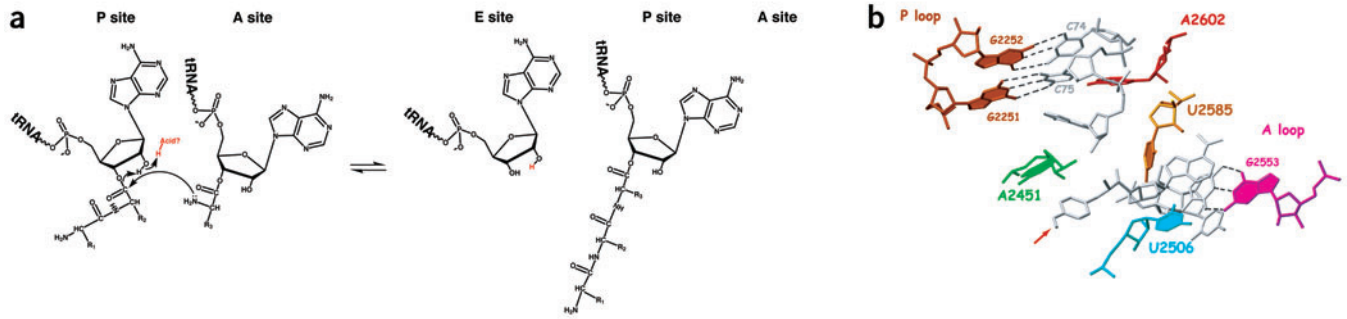

Figure 3 Possible mechanism for peptide bond formation catalyzed by the large subunit of the ribosome. (**a**) The amino group of an aminoacylated substrate tRNA in the ribosomal A site attacks the carbonyl carbon at the terminus of the tRNA esterified to the growing peptide chain in the ribosomal P site, producing an new amide (peptide) bond and an alcohol (the deacylated tRNA). The end of the spent tRNA previously in the P site moves into the E site and that of the tRNA containing the growing peptide chain moves into the P site. In the mechanism shown, the 2′ OH at the end of the P-site tRNA acts as an intermediary to donate a proton to the leaving group 3′ oxygen. (**b**) A view of the active site of the ribosome[106] (PDB entry 1KQS). The A and P loops (purple and brown, respectively) interact with the 3′ ends of the substrate tRNAs (gray), orienting the attached amino acid (highlighted by red arrow) and peptide (not shown) for bond formation. The active site residues A2451, U2506, U2585 and A2602 are all positioned near the site of chemistry. However, mutation of these bases has no effect on the rate of peptide bond formation with intact tRNA substrates[98]. **Figure 3b** was prepared by S. Dorner using Ribbons (http://sgce.cbse.uab.edu/ribbons/).

The first detailed view of a group I intron was provided by the *T. thermophila* P4-P6 domain crystal structure, the first crystal structure to show how RNA-RNA and divalent cation-mediated contacts stabilize a globular fold with a solvent-inaccessible interior[76]. Although comparative phylogenetic analysis[77] and a modest-resolution crystal structure of a truncated ribozyme[78] gave insights into the global architecture of the intact intron, crystal structures of group I introns from *T. thermophila*, *Azoarcus* and the Twort phage have now revealed in exciting detail how the active site is arranged[79–81] (**Fig. 2d**). All the structures show that binding of the essential guanosine c-factor is achieved through interactions within a specific binding pocket created by five conserved residues within the P7 stem. The *Azoarcus* structure at a resolution of 3.1 Å of a 'trapped' complex containing 2′-deoxy residues in the substrate strand to prevent splicing also reveals binding sites for two divalent metal ions located tantalizingly close to the site of action (**Fig. 2d**, middle). Although consistent with earlier predictions of a two-metal-ion mechanism similar to those of polymerases[82], these structural data are seemingly at odds with the three-metal-ion model derived from thiophilic metal ion rescue experiments. In the structure at a resolution of 3.6 Å of the phage Twort group I intron in complex with a product analog, only one metal ion is observed in the active site, corresponding to the biochemically identified metal ion A[81] (**Fig. 2d**, right). The failure to observe other metal ions may be the result of the high Li^+ concentration required for crystallization. In contrast to the *Azoarcus* structure, both metal ions B and C can be modeled into the active site of the Twort structure in positions appropriate for the metals' proposed roles in catalysis and consistent with their putative ligands on the ribozyme. There are subtle differences in the conformations of the guanosine-binding sites in the *Azoarcus* and Twort structures, which may lead to the discrepancy in the positions of the bound metals. These differences might be due to the lack of a 2′ hydroxyl on the guanosine in the binding site in the *Azoarcus* structure, because this group seems to make structurally important contacts in the Twort structure that induce a more open conformation in the active site. In the *T. thermophila* structure, crystallized in the absence of its RNA substrate, the single metal ion observed in the active site is located between the positions of the two metals seen in the *Azoarcus* structure, implying possible conformational adjustments upon substrate docking[80] (**Fig. 2d**, left).

Though crystallographic structures can reveal the precise locations and identities of metal ions within a molecule, a single structure does not reflect all of the states of a reaction cycle. Chemical substitution and thiophilic metal ion rescue experiments are a powerful method for probing the locations and functions of catalytic metal ions, but they run the danger of creating non-native ion-binding sites[83]. Thus, resolution of the group I intron reaction mechanism awaits further structures and biochemical analyses to sort out these possibilities. Whatever the answer, one thing is clear: the ribozyme field seems to be learning what the protein enzymology community has long known—that three-dimensional structures are extremely valuable, but very rarely close a chapter, let alone the whole book, on the mechanism of action of an enzyme.

Peptide bond formation

According to the RNA world hypothesis, ribozymes that once dominated a primitive metabolism were largely supplanted by more efficient protein enzymes in the course of evolution. Intriguingly, however, the catalyst still responsible for synthesizing nearly all proteins in cells is in fact a ribozyme. Though a few specialized peptides, mostly antibiotics, are made by protein enzymes[84–86], the vast majority of proteins are synthesized by the ribosome, a ribonucleoprotein machine that conducts information-directed protein synthesis in all of life. Biochemical evidence for a primary role of the RNA in this activity[87–91] was bolstered by the discovery of an all-RNA active site in the peptidyl transferase center of the large ribosomal subunit[92]. This exciting finding focused attention on the chemical mechanism of peptide bond formation because for the first time it was possible to interpret the catalytic consequences of small changes in the ribosomal active site.

The ribosome translates the information contained in a template mRNA into the encoded polypeptide using two different substrate tRNAs, one with the growing peptide chain attached by an ester linkage to its 3′ hydroxyl (the P site or peptidyl tRNA), and the other with a single amino acid esterified to its 3′ hydroxyl (the A site or acceptor tRNA). During peptide bond formation, the amine on the A-site aminoacyl tRNA attacks the carbonyl carbon of the P-site peptidyl tRNA to produce an amide and an alcohol (**Fig. 3**). To accelerate this reaction by the observed ~10^7-fold above the uncatalyzed rate[93], the ribosome could use a variety of different strategies, from general acid-base catalysis to

simply orienting the substrates in the appropriate configuration[93] to achieve the transition state.

One approach to determining the catalytic mechanism of the ribosome was to cocrystallize the peptidyl transferase–containing 50S subunit of the ribosome with a small molecule inhibitor of the ribosome, an analog of the anionic tetrahedral intermediate in amide bond formation[94]. The proximity of the inhibitor to conserved active site nucleotides led to the hypothesis that A2451 acts as a general base to abstract an amino proton from the incoming amino acid[94,95].

One of the challenges in studying the molecular mechanism used by ribosomes in catalyzing peptide bond formation is the presence of multiple genes encoding ribosomal RNAs in bacterial cells, which, coupled with the requirement of functional ribosomes for life, made it impossible to make pure populations of ribosomes with deleterious mutations in their rRNAs[96]. This problem was partially circumvented by purifying mutant ribosomes from a strain also expressing the wild-type version and then deconvoluting the contribution of the wild-type and mutant ribosomes to pre-steady state kinetic data. In this manner, it was shown that changing A2451 to a U reduced the rate of peptide bond formation with the A-site tRNA analog puromycin and shifted one of the two observed pK_a values derived from rate versus pH profiles, consistent with the proposed role of A2451 in acid-base catalysis[97].

More recently, techniques have been developed for purifying rRNA affinity-tagged mutant ribosomes. Using this approach, ribosomes bearing all possible single mutations at the four nucleotide positions surrounding the catalytic site were purified and studied[98]. Pre-steady-state kinetic analysis of the reaction between a P site–bound dipeptidyl tRNA and puromycin showed that all but 1 of the 12 mutants that were studied caused a substantial (30–9,400-fold) reduction in the rate of peptide bond formation. Strikingly, however, none of these mutant ribosomes showed any defect in the rate of peptide bond formation when the A-site substrate was instead an intact aminoacyl tRNA. Though in principle the chemical step with the natural A-site tRNA substrate might be faster than the rate-limiting step of the reaction, masking direct effects of the mutations on catalysis, this would imply rates of peptide bond formation that are 10^4-fold faster than the rate of polypeptide elongation *in vivo*[99]. Although no defects were observed in peptide bond formation in this assay, mutations of several of the universally conserved nucleotides were found to diminish the rate of peptide chain release during the termination phase of protein synthesis. Thus the role of these active site residues may not be to facilitate the relatively easy attack of an amine on an activated ester during peptide bond formation, but may instead be to activate water for the more difficult hydrolysis of the completed protein from the final P-site tRNA. The rate defects observed with puromycin probably reflect deficiencies in the positioning of this weakly bound substrate analog, arguing that orientation of the reactive groups is a critical aspect of catalysis by the ribosome.

In addition to substrate positioning, the P-site tRNA substrate itself might contribute to catalysis of peptide bond formation. Evidence for an active role for the tRNA comes from recent experiments in which the 2′ hydroxyl on the last nucleotide of the P-site tRNA was substituted with a hydrogen or fluorine atom[100]. In either case, the substitution reduced the rate of peptide bond formation by $\geq 10^6$-fold under conditions in which the chemical step of the reaction (rather than substrate binding or a conformational change) is thought to be rate-limiting. This could mean that this single 2′ OH group contributes the lion's share of catalysis on the ribosome, either directly or by bringing in another group such as a metal ion or water. As usual, however, there are alternate possibilities to explain these observations. For instance, the modifications might cause the substrate to bind in a nonproductive mode or the 2′ hydroxyl, although not contributing directly to stabilization of the transition state, might be part of a required pathway for proton movement into or out of the active site and blocking this pathway would create a new, very large energy barrier.

Conclusions

The growing number of high-resolution ribozyme structures, coupled with detailed biochemical studies, has shed much light on the basis for RNA-mediated catalysis, although many shadows and dark corners remain. Perhaps the most general, and in hindsight not surprising, point that emerges from this impressive body of work is that ribozymes catalyze reactions in the same ways that proteins do: they form substrate-binding sites to decrease the entropic cost of attaining the transition state, they have more favorable interactions with the transition state structure than with the ground state, they facilitate the movement of protons during the reaction, and they can even raise the energy of the bound substrate relative to the transition state. In contrast to what had been proposed about them early on, ribozymes are not limited to using only metal ions as functional groups in catalysis, but can also use nucleotide bases, sugar hydroxyls and even the phosphate backbone. Because of their limited side chain repertoire and possibly also their tendency to flop around, ribozymes are, in the end, not as adept at catalyzing a wide variety of reactions as are protein enzymes, and thus nature has favored amino acid–based catalysts over RNA-based ones. Nonetheless, ribozymes are still extant and the lives of all organisms seem to depend on them. The recent discoveries of ribozymes involved in regulating gene expression in both bacteria and eukaryotes (riboswitches) highlight their continued importance in modern biology[101,102].

Understanding the specific roles of metal ions in ribozyme catalysis, the prevalence (or scarcity) of general acid-base catalysis by RNA and the relative contributions of various catalytic strategies to observed ribozyme rate enhancements will make it increasingly possible to compare the details of RNA- and protein-based catalysis. Such comparisons will help illuminate the reasons that ribozymes occupy indispensable niches in modern biology as well as provide insight into the catalytic roles RNA may play within complex ribonucleoproteins such as the spliceosome.

ACKNOWLEDGMENTS

The authors thank E. Friedman, K. Karbstein and A. Ke for helpful comments on the manuscript, R. Green and A. Mildvan for stimulating discussions, and A. Ke and S. Dorner for preparation of **Figures 1b**,**d**, **2b**,**d** and **3b**.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 6 January; accepted 5 April 2005
Published online at http://www.nature.com/nsmb/

1. Jencks, W.P. *Catalysis in Chemistry and Enzymology*, 864 (Dover Publications, New York, 1987).
2. Narlikar, G.J. & Herschlag, D. Mechanistic aspects of enzymatic catalysis: lessons from comparison of RNA and protein enzymes. *Annu. Rev. Biochem.* **66**, 19–59 (1997).
3. Armstrong, A.A. & Amzel, L.M. Role of entropy in increased rates of intramolecular reactions. *J. Am. Chem. Soc.* **125**, 14596–14602 (2003).
4. Fersht, A.R. Catalysis, binding and enzyme-substrate complementarity. *Proc. R. Soc. Lond. B. Biol. Sci.* **187**, 397–407 (1974).
5. Jencks, W.P. Imidazole and proton transfer in catalysis. *Biochem. Soc. Symp.* **31**, 59–80 (1970).
6. Tesmer, J.J. *et al.* Two-metal-ion catalysis in adenylyl cyclase. *Science* **285**, 756–760 (1999).
7. Wyckoff, H.W. *et al.* The three-dimensional structure of ribonuclease-S. Interpretation of an electron density map at a nominal resolution of 2 Å. *J. Biol. Chem.* **245**, 305–328 (1970).
8. Drum, C.L. *et al.* Structural basis for the activation of anthrax adenylyl cyclase exotoxin by calmodulin. *Nature* **415**, 396–402 (2002).
9. Tang, J. & Breaker, R.R. Structural diversity of self-cleaving ribozymes. *Proc. Natl. Acad. Sci. USA* **97**, 5784–5789 (2000).
10. Salehi-Ashtiani, K. & Szostak, J.W. *In vitro* evolution suggests multiple origins for the hammerhead ribozyme. *Nature* **414**, 82–84 (2001).

11. Khvorova, A., Lescoute, A., Westhof, E. & Jayasena, S.D. Sequence elements outside the hammerhead ribozyme catalytic core enable intracellular activity. *Nat. Struct. Biol.* **10**, 708–712 (2003).
12. Canny, M.D. *et al.* Fast cleavage kinetics of a natural hammerhead ribozyme. *J. Am. Chem. Soc.* **126**, 10848–10849 (2004).
13. Penedo, J.C., Wilson, T.J., Jayasena, S.D., Khvorova, A. & Lilley, D.M. Folding of the natural hammerhead ribozyme is enhanced by interaction of auxiliary elements. *RNA* **10**, 880–888 (2004).
14. Stage-Zimmermann, T.K. & Uhlenbeck, O.C. Hammerhead ribozyme kinetics. *RNA* **4**, 875–889 (1998).
15. Raines, R.T. Active site of ribonuclease A. In *Nucleic Acids and Molecular Biology* Vol. 13 (ed. Zenkova, M.A.) (Springer, Heidelberg, Germany, 2004).
16. Murray, J.B., Seyhan, A.A., Walter, N.G., Burke, J.M. & Scott, W.G. The hammerhead, hairpin and VS ribozymes are catalytically proficient in monovalent cations alone. *Chem. Biol.* **5**, 587–595 (1998).
17. Curtis, E.A. & Bartel, D.P. The hammerhead cleavage reaction in monovalent cations. *RNA* **7**, 546–552 (2001).
18. O'Rear, J.L. *et al.* Comparison of the hammerhead cleavage reactions stimulated by monovalent and divalent cations. *RNA* **7**, 537–545 (2001).
19. Pley, H.W., Flaherty, K.M. & McKay, D.B. Three-dimensional structure of a hammerhead ribozyme. *Nature* **372**, 68–74 (1994).
20. Scott, W.G., Finch, J.T. & Klug, A. The crystal structure of an all-RNA hammerhead ribozyme: a proposed mechanism for RNA catalytic cleavage. *Cell* **81**, 991–1002 (1995).
21. Scott, W.G., Murray, J.B., Arnold, J.R., Stoddard, B.L. & Klug, A. Capturing the structure of a catalytic RNA intermediate: the hammerhead ribozyme. *Science* **274**, 2065–2069 (1996).
22. Murray, J.B. *et al.* The structural basis of hammerhead ribozyme self-cleavage. *Cell* **92**, 665–673 (1998).
23. Murray, J.B., Szoke, H., Szoke, A. & Scott, W.G. Capture and visualization of a catalytic RNA enzyme-product complex using crystal lattice trapping and X-ray holographic reconstruction. *Mol. Cell* **5**, 279–287 (2000).
24. Murray, J.B., Dunham, C.M. & Scott, W.G. A pH-dependent conformational change, rather than the chemical step, appears to be rate-limiting in the hammerhead ribozyme cleavage reaction. *J. Mol. Biol.* **315**, 121–130 (2002).
25. Dunham, C.M., Murray, J.B. & Scott, W.G. A helical twist-induced conformational switch activates cleavage in the hammerhead ribozyme. *J. Mol. Biol.* **332**, 327–336 (2003).
26. Ruffner, D.E. & Uhlenbeck, O.C. Thiophosphate interference experiments locate phosphates important for the hammerhead RNA self-cleavage reaction. *Nucleic Acids Res.* **18**, 6025–6029 (1990).
27. Peracchi, A., Beigelman, L., Scott, E.C., Uhlenbeck, O.C. & Herschlag, D. Involvement of a specific metal ion in the transition of the hammerhead ribozyme to its catalytic conformation. *J. Biol. Chem.* **272**, 26822–26826 (1997).
28. Scott, E.C. & Uhlenbeck, O.C. A re-investigation of the thio effect at the hammerhead cleavage site. *Nucleic Acids Res.* **27**, 479–484 (1999).
29. Wang, S., Karbstein, K., Peracchi, A., Beigelman, L. & Herschlag, D. Identification of the hammerhead ribozyme metal ion binding site responsible for rescue of the deleterious effect of a cleavage site phosphorothioate. *Biochemistry* **38**, 14363–14378 (1999).
30. Murray, J.B. & Scott, W.G. Does a single metal ion bridge the A-9 and scissile phosphate groups in the catalytically active hammerhead ribozyme structure? *J. Mol. Biol.* **296**, 33–41 (2000).
31. Ferre-D'Amare, A.R., Zhou, K. & Doudna, J.A. Crystal structure of a hepatitis δ virus ribozyme. *Nature* **395**, 567–574 (1998).
32. Rupert, P.B. & Ferre-D'Amare, A.R. Crystal structure of a hairpin ribozyme-inhibitor complex with implications for catalysis. *Nature* **410**, 780–786 (2001).
33. Bevilacqua, P.C. Mechanistic considerations for general acid-base catalysis by RNA: revisiting the mechanism of the hairpin ribozyme. *Biochemistry* **42**, 2259–2265 (2003).
34. Rajagopal, P. & Feigon, J. Triple-strand formation in the homopurine:homopyrimidine DNA oligonucleotides d(G-A)4 and d(T-C)4. *Nature* **339**, 637–640 (1989).
35. Sklenar, V. & Feigon, J. Formation of a stable triplex from a single DNA strand. *Nature* **345**, 836–838 (1990).
36. Connell, G.J. & Yarus, M. RNAs with dual specificity and dual RNAs with similar specificity. *Science* **264**, 1137–1141 (1994).
37. Legault, P. & Pardi, A. In situ probing of adenine protonation in RNA by 13C NMR. *J. Am. Chem. Soc.* **116**, 8390–8391 (1994).
38. Ravindranathan, S., Butcher, S.E. & Feigon, J. Adenine protonation in domain B of the hairpin ribozyme. *Biochemistry* **39**, 16026–16032 (2000).
39. Been, M.D. & Wickham, G.S. Self-cleaving ribozymes of hepatitis δ virus RNA. *Eur. J. Biochem.* **247**, 741–753 (1997).
40. Thompson, J.E., Venegas, F.D. & Raines, R.T. Energetics of catalysis by ribonucleases: fate of the 2′,3′-cyclic phosphodiester intermediate. *Biochemistry* **33**, 7408–7414 (1994).
41. Lebruska, L.L., Kuzmine, II & Fedor, M.J. Rescue of an abasic hairpin ribozyme by cationic nucleobases: evidence for a novel mechanism of RNA catalysis. *Chem. Biol.* **9**, 465–473 (2002).
42. Perrotta, A.T., Shih, I. & Been, M.D. Imidazole rescue of a cytosine mutation in a self-cleaving ribozyme. *Science* **286**, 123–126 (1999).
43. Shih, I.H. & Been, M.D. Involvement of a cytosine side chain in proton transfer in the rate-determining step of ribozyme self-cleavage. *Proc. Natl. Acad. Sci. USA* **98**, 1489–1494 (2001).
44. Nakano, S., Chadalavada, D.M. & Bevilacqua, P.C. General acid-base catalysis in the mechanism of a hepatitis δ virus ribozyme. *Science* **287**, 1493–1497 (2000).
45. Nakano, S., Proctor, D.J. & Bevilacqua, P.C. Mechanistic characterization of the HDV genomic ribozyme: assessing the catalytic and structural contributions of divalent metal ions within a multichannel reaction mechanism. *Biochemistry* **40**, 12022–12038 (2001).
46. Nakano, S. & Bevilacqua, P.C. Proton inventory of the genomic HDV ribozyme in Mg(2+)-containing solutions. *J. Am. Chem. Soc.* **123**, 11333–11334 (2001).
47. Luptak, A., Ferre-D'Amare, A.R., Zhou, K., Zilm, K.W. & Doudna, J.A. Direct pK(a) measurement of the active-site cytosine in a genomic hepatitis δ virus ribozyme. *J. Am. Chem. Soc.* **123**, 8447–8452 (2001).
48. Ke, A., Zhou, K., Ding, F., Cate, J.H. & Doudna, J.A. A conformational switch controls hepatitis δ virus ribozyme catalysis. *Nature* **429**, 201–205 (2004).
49. Kuzmin, Y.I., Da Costa, C.P. & Fedor, M.J. Role of an active site guanine in hairpin ribozyme catalysis probed by exogenous nucleobase rescue. *J. Mol. Biol.* **340**, 233–251 (2004).
50. Nahas, M.K. *et al.* Observation of internal cleavage and ligation reactions of a ribozyme. *Nat. Struct. Mol. Biol.* **11**, 1107–1113 (2004).
51. Rupert, P.B., Massey, A.P., Sigurdsson, S.T. & Ferre-D'Amare, A.R. Transition state stabilization by a catalytic RNA. *Science* **298**, 1421–1424 (2002).
52. Ryder, S.P. *et al.* Investigation of adenosine base ionization in the hairpin ribozyme by nucleotide analog interference mapping. *RNA* **7**, 1454–1463 (2001).
53. Hampel, A. & Cowan, J.A. A unique mechanism for RNA catalysis: the role of metal cofactors in hairpin ribozyme cleavage. *Chem. Biol.* **4**, 513–517 (1997).
54. Nesbitt, S., Hegg, L.A. & Fedor, M.J. An unusual pH-independent and metal-ion-independent mechanism for hairpin ribozyme catalysis. *Chem. Biol.* **4**, 619–630 (1997).
55. Hampel, K.J. & Burke, J.M. A conformational change in the "loop E-like" motif of the hairpin ribozyme is coincidental with domain docking and is essential for catalysis. *Biochemistry* **40**, 3723–3729 (2001).
56. Blount, K.F., Grover, N.L., Mokler, V., Beigelman, L. & Uhlenbeck, O.C. Steric interference modification of the hammerhead ribozyme. *Chem. Biol.* **9**, 1009–1016 (2002).
57. Pereira, M.J., Harris, D.A., Rueda, D. & Walter, N.G. Reaction pathway of the trans-acting hepatitis δ virus ribozyme: a conformational change accompanies catalysis. *Biochemistry* **41**, 730–740 (2002).
58. Hampel, K.J. & Burke, J.M. Solvent protection of the hammerhead ribozyme in the ground state: evidence for a cation-assisted conformational change leading to catalysis. *Biochemistry* **42**, 4421–4429 (2003).
59. Doherty, E.A., Herschlag, D. & Doudna, J.A. Assembly of an exceptionally stable RNA tertiary interface in a group I ribozyme. *Biochemistry* **38**, 2982–2990 (1999).
60. Engelhardt, M.A., Doherty, E.A., Knitt, D.S., Doudna, J.A. & Herschlag, D. The P5abc peripheral element facilitates preorganization of the *Tetrahymena* group I ribozyme for catalysis. *Biochemistry* **39**, 2639–2651 (2000).
61. Ohuchi, S.J., Ikawa, Y., Shiraishi, H. & Inoue, T. Modular engineering of a Group I intron ribozyme. *Nucleic Acids Res.* **30**, 3473–3480 (2002).
62. De la Pena, M., Gago, S. & Flores, R. Peripheral regions of natural hammerhead ribozymes greatly increase their self-cleavage activity. *EMBO J.* **22**, 5561–5570 (2003).
63. Costa, M., Michel, F. & Westhof, E. A three-dimensional perspective on exon binding by a group II self-splicing intron. *EMBO J.* **19**, 5007–5018 (2000).
64. Swisher, J., Duarte, C.M., Su, L.J. & Pyle, A.M. Visualizing the solvent-inaccessible core of a group II intron ribozyme. *EMBO J.* **20**, 2051–2061 (2001).
65. Zhang, L. & Doudna, J.A. Structural insights into group II intron catalysis and branch-site selection. *Science* **295**, 2084–2088 (2002).
66. Sigel, R.K. *et al.* Solution structure of domain 5 of a group II intron ribozyme reveals a new RNA motif. *Nat. Struct. Mol. Biol.* **11**, 187–192 (2004).
67. Herschlag, D. & Cech, T.R. Catalysis of RNA cleavage by the *Tetrahymena thermophila* ribozyme. 1. Kinetic description of the reaction of an RNA substrate complementary to the active site. *Biochemistry* **29**, 10159–10171 (1990).
68. Herschlag, D. & Cech, T.R. Catalysis of RNA cleavage by the *Tetrahymena thermophila* ribozyme. 2. Kinetic description of the reaction of an RNA substrate that forms a mismatch at the active site. *Biochemistry* **29**, 10172–10180 (1990).
69. McSwiggen, J.A. & Cech, T.R. Stereochemistry of RNA cleavage by the *Tetrahymena* ribozyme and evidence that the chemical step is not rate-limiting. *Science* **244**, 679–683 (1989).
70. Rajagopal, J., Doudna, J.A. & Szostak, J.W. Stereochemical course of catalysis by the *Tetrahymena* ribozyme. *Science* **244**, 692–694 (1989).
71. Shan, S., Yoshida, A., Sun, S., Piccirilli, J.A. & Herschlag, D. Three metal ions at the active site of the *Tetrahymena* group I ribozyme. *Proc. Natl. Acad. Sci. USA* **96**, 12299–12304 (1999).
72. Shan, S.O. & Herschlag, D. Probing the role of metal ions in RNA catalysis: kinetic and thermodynamic characterization of a metal ion interaction with the 2′-moiety of the guanosine nucleophile in the *Tetrahymena* group I ribozyme. *Biochemistry* **38**, 10958–10975 (1999).
73. Shan, S., Kravchuk, A.V., Piccirilli, J.A. & Herschlag, D. Defining the catalytic metal ion interactions in the *Tetrahymena* ribozyme reaction. *Biochemistry* **40**, 5161–5171 (2001).
74. Piccirilli, J.A., Vyle, J.S., Caruthers, M.H. & Cech, T.R. Metal ion catalysis in the *Tetrahymena* ribozyme reaction. *Nature* **361**, 85–88 (1993).
75. Narlikar, G.J., Gopalakrishnan, V., McConnell, T.S., Usman, N. & Herschlag, D. Use of binding energy by an RNA enzyme for catalysis by positioning and substrate destabilization. *Proc. Natl. Acad. Sci. USA* **92**, 3668–3672 (1995).
76. Cate, J.H. *et al.* Crystal structure of a group I ribozyme domain: principles of RNA

packing. *Science* **273**, 1678–1685 (1996).
77. Lehnert, V., Jaeger, L., Michel, F. & Westhof, E. New loop-loop tertiary interactions in self-splicing introns of subgroup IC and ID: a complete 3D model of the *Tetrahymena thermophila* ribozyme. *Chem. Biol.* **3**, 993–1009 (1996).
78. Golden, B.L., Gooding, A.R., Podell, E.R. & Cech, T.R. A preorganized active site in the crystal structure of the *Tetrahymena* ribozyme. *Science* **282**, 259–264 (1998).
79. Adams, P.L., Stahley, M.R., Kosek, A.B., Wang, J. & Strobel, S.A. Crystal structure of a self-splicing group I intron with both exons. *Nature* **430**, 45–50 (2004).
80. Guo, F., Gooding, A.R. & Cech, T.R. Structure of the *Tetrahymena* ribozyme: base triple sandwich and metal ion at the active site. *Mol. Cell* **16**, 351–362 (2004).
81. Golden, B.L., Kim, H. & Chase, E. Crystal structure of a phage Twort group I ribozyme–product complex. *Nat. Struct. Mol. Biol.* **12**, 82–89 (2005).
82. Steitz, T.A. & Steitz, J.A. A general two-metal-ion mechanism for catalytic RNA. *Proc. Natl. Acad. Sci. USA* **90**, 6498–6502 (1993).
83. Vortler, L.C. & Eckstein, F. Phosphorothioate modification of RNA for stereochemical and interference analyses. *Methods Enzymol.* **317**, 74–91 (2000).
84. Keating, T.A. & Walsh, C.T. Initiation, elongation, and termination strategies in polyketide and polypeptide antibiotic biosynthesis. *Curr. Opin. Chem. Biol.* **3**, 598–606 (1999).
85. Sieber, S.A. & Marahiel, M.A. Learning from nature's drug factories: nonribosomal synthesis of macrocyclic peptides. *J. Bacteriol.* **185**, 7036–7043 (2003).
86. Walsh, C.T. Polyketide and nonribosomal peptide antibiotics: modularity and versatility. *Science* **303**, 1805–1810 (2004).
87. Moazed, D. & Noller, H.F. Interaction of tRNA with 23S rRNA in the ribosomal A, P, and E sites. *Cell* **57**, 585–597 (1989).
88. Noller, H.F., Hoffarth, V. & Zimniak, L. Unusual resistance of peptidyl transferase to protein extraction procedures. *Science* **256**, 1416–1419 (1992).
89. Samaha, R.R., Green, R. & Noller, H.F. A base pair between tRNA and 23S rRNA in the peptidyl transferase centre of the ribosome. *Nature* **377**, 309–314 (1995).
90. Green, R., Samaha, R.R. & Noller, H.F. Mutations at nucleotides G2251 and U2585 of 23 S rRNA perturb the peptidyl transferase center of the ribosome. *J. Mol. Biol.* **266**, 40–50 (1997).
91. Green, R., Switzer, C. & Noller, H.F. Ribosome-catalyzed peptide-bond formation with an A-site substrate covalently linked to 23S ribosomal RNA. *Science* **280**, 286–289 (1998).
92. Ban, N., Nissen, P., Hansen, J., Moore, P.B. & Steitz, T.A. The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution. *Science* **289**, 905–920 (2000).
93. Sievers, A., Beringer, M., Rodnina, M.V. & Wolfenden, R. The ribosome as an entropy trap. *Proc. Natl. Acad. Sci. USA* **101**, 7897–7901 (2004).
94. Nissen, P., Hansen, J., Ban, N., Moore, P.B. & Steitz, T.A. The structural basis of ribosome activity in peptide bond synthesis. *Science* **289**, 920–930 (2000).
95. Muth, G.W., Ortoleva-Donnelly, L. & Strobel, S.A. A single adenosine with a neutral pKa in the ribosomal peptidyl transferase center. *Science* **289**, 947–950 (2000).
96. Asai, T. *et al.* Construction and initial characterization of *Escherichia coli* strains with few or no intact chromosomal rRNA operons. *J. Bacteriol.* **181**, 3803–3809 (1999).
97. Katunin, V.I., Muth, G.W., Strobel, S.A., Wintermeyer, W. & Rodnina, M.V. Important contribution to catalysis of peptide bond formation by a single ionizing group within the ribosome. *Mol. Cell* **10**, 339–346 (2002).
98. Youngman, E.M., Brunelle, J.L., Kochaniak, A.B. & Green, R. The active site of the ribosome is composed of two layers of conserved nucleotides with distinct roles in peptide bond formation and peptide release. *Cell* **117**, 589–599 (2004).
99. Pedersen, S. *Escherichia coli* ribosomes translate *in vivo* with variable rate. *EMBO J.* **3**, 2895–2898 (1984).
100. Weinger, J.S., Parnell, K.M., Dorner, S., Green, R. & Strobel, S.A. Substrate-assisted catalysis of peptide bond formation by the ribosome. *Nat. Struct. Mol. Biol.* **11**, 1101–1106 (2004).
101. Winkler, W.C., Nahvi, A., Roth, A., Collins, J.A. & Breaker, R.R. Control of gene expression by a natural metabolite-responsive ribozyme. *Nature* **428**, 281–286 (2004).
102. Teixeira, A. *et al.* Autocatalytic RNA cleavage in the human β-globin pre-mRNA promotes transcription termination. *Nature* **432**, 526–530 (2004).
103. Xu, R.X. *et al.* Atomic structure of PDE4: insights into phosphodiesterase mechanism and specificity. *Science* **288**, 1822–1825 (2000).
104. Mildvan, A.S. *et al.* Structures and mechanisms of Nudix hydrolases. *Arch. Biochem. Biophys.* **433**, 129–143 (2005).
105. Huai, Q., Colicelli, J. & Ke, H. The crystal structure of AMP-bound PDE4 suggests a mechanism for phosphodiesterase catalysis. *Biochemistry* **42**, 13220–13226 (2003).
106. Schmeing, T.M. *et al.* A pre-translocational intermediate in protein synthesis observed in crystals of enzymatically active 50S subunits. *Nat. Struct. Biol.* **9**, 225–230 (2002).