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Critical Review

Insights into Functional Modulation of Catalytic RNA Activity

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Summary

RNA molecules play critical roles in cell biology, and novel findings continuously broaden their functional repertoires. Apart from their well-documented participation in protein synthesis, it is now apparent that several noncoding RNAs (*i.e.*, micro-RNAs and riboswitches) also participate in the regulation of gene expression. The discovery of catalytic RNAs had profound implications on our views concerning the evolution of life on our planet at a molecular level. A characteristic attribute of RNA, probably traced back to its ancestral origin, is the ability to interact with and be modulated by several ions and molecules of different sizes. The inhibition of ribosome activity by antibiotics has been extensively used as a therapeutical approach, while activation and substrate-specificity alteration have the potential to enhance the versatility of ribozyme-based tools in translational research. In this review, we will describe some representative examples of such modulators to illustrate the potential of catalytic RNAs as tools and targets in research and clinical approaches.

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Keywords ribozymes; RNase P; ribosome; hammerhead; hairpin; group I intron; antibiotics; modulators.

INTRODUCTION

Significant discoveries made during the past 25 years have changed scientific views of RNA from an essentially passive intermediate of the flow of genetic information to an important player assuming central roles in the cell. The discovery that RNA molecules can act as genuine biological catalysts founded the 'RNA world' hypothesis, which is now considered a plausible scenario for the emergence of life on our planet: the first 'living' system comprised self-replicases composed of RNA (*1*). RNA can be both informational and catalytic and therefore can provide a logical solution to the 'chicken and egg' type of problem concerning the first living systems at a molecular level. It is worth mentioning that the idea that the RNA was the first biomolecule was presciently formulated in two famous papers by Crick and Orgel in the late 1960s (*2, 3*).

RNA catalysts and other 'functional' RNAs (*e.g.*, riboswitches) can naturally or artificially evolve into flexible 'biosensors' that can modulate their activity by interacting with a wide variety of molecules (*4*). It is considered that such molecules (*e.g.*, peptides and antibiotics) could have been present in prebiotic conditions (*5*) assisting primordial reactions. Most probably, such molecules provided additional functional groups to the chemical repertoire of ribozymes (*6*) and extended the structural capabilities of RNA molecules allowing them to form novel three-dimensional shapes (*7*). In the contemporary world, proteins are common partners of RNA, in general, and ribozymes, in particular, as many of them naturally form ribonucleoprotein complexes *in vivo*. The two universally conserved representatives of this group are the ribosome and ribonuclease P (RNase P). The RNA subunit of RNase P can catalyse the 5′ maturation of precursor tRNA *in vitro* in the absence of proteins (*8–10*), while crystallographic studies of the ribosome have provided evidence that the peptidyl-transferase centre at least in some species is composed solely of RNA (*11*). Other examples of RNA catalysts include the hammerhead, hepatitis delta virus (HDV), hairpin self-cleaving RNAs and group I self-splicing introns.

Naturally occurring ribozymes (apart from ribosomes) catalyse phosphate group transfer, adopting two reaction mechanisms that differ in their products (*12*). The small self-cleaving RNAs catalyse phosphodiester cleavage reactions that generate 5′-hydroxyl and 2′–3′-cyclic-phosphate termini. Large ribozymes, namely the trans acting RNase P and self-splicing introns catalyse phosphodiester-cleavage ligation reactions that produce 5′-phosphate and 3′-hydroxyl termini.

RNAs can form compact and ordered structures needed for active site architecture, as evidenced by crystal structures

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(*13–15*). Nevertheless, in contrast to protein enzymes, which contain chemically versatile amino acids, RNA is composed of only four different nucleotides with apparently limited capabilities of providing useful functional groups for their enzymatic centres. Nucleobases do not ionise significantly at pH values 5–9, meaning that they do not readily accept or donate protons at neutral pH. Furthermore, unless there is a significant perturbation of pK_a values, no functional groups carry a positive charge at neutral pH, which is required for the stabilization of an electronegative transition state. It has been suggested that metal-ion cofactors could assist the functional groups of RNA to accomplish these objectives (*16*).

Ribozymes can effectively recognise and cleave-specific RNA sequences *in vivo* and *in vitro* (*17*). Moreover, RNAs and RNA-protein complexes from pathogens are common therapeutical targets (*e.g.*, ribosome is one of the main antibiotic targets in the cell) (*18*). Clearly, the study of functional RNAs and, more particularly, ribozymes advances our knowledge on the evolutionary history of biomolecules and contributes to the development of research and therapeutical tools.

This review is meant to summarise in a comprehensive way our current knowledge on strategies of ribozyme modulation in an effort to illustrate their multifacet characters.

MODULATION OF RIBOSOMAL ACTIVITY

The ribosome is the most abundant ribozyme in nature and is the only RNA-based polymerase. Ribosomal RNA is involved in all steps of protein synthesis, that is, binding of translation factors, binding of mRNA, subunit association, positioning and translocation of tRNA substrates, recognition of cognate tRNA and peptidyl transferase (PTase) activity. On the basis of biochemical and genetic studies, 23S rRNA has long been suspected to be the functional component of the PTase centre (*19, 20*). This was convincingly demonstrated when the high-resolution structure of 50S subunit showed that there is no protein in the active site where peptide bond formation occurs in the *Haloarcula martismortui* ribosome (*11*). However, recent studies have shown that the L27 protein of the large ribosomal subunit extends its amino terminus into the peptidyl-transferase centre, at least in *E. coli*. Absence of this protein has a detrimental effect on the activity status of PTase. Thus, L27 could be considered as a modulator of PTase activity (*21*). In the following section, we will focus on the effect of small molecule effectors that target 23S rRNA.

The importance of polyamines in the RNA function relies in the fact that the majority are found in mammalian or bacterial cells to be bound with RNA molecules. Polyamines (Fig. 1A) exert their polycationic characters as components of the ionic environment of the ribosomes.

The modulation of peptidyl-transferase by polyamines has been extensively studied by kinetic analysis and has been found that spermine, depending on its concentration, can cause such conformational changes leading to the activation or inhibition of the peptide bond formation (*22, 23*). Studies using a photoreactive analogue of spermine, ABA-spermine, have shown that spermine binds to several sites of 23S rRNA that are located into the PTase centre (*24*) as well as with the ribosomal proteins L2, L3, L6, L15, L17, and L18 that are topographically adjacent to the PTase centre (*25, 26*). It was speculated that polyamines may influence the conformation of specific 'PTase proteins', which, in turn, affect the conformation of ribosomal RNA residues involved in the catalysis of peptide bond formation. It was hypothesised that binding of polyamines to the above proteins

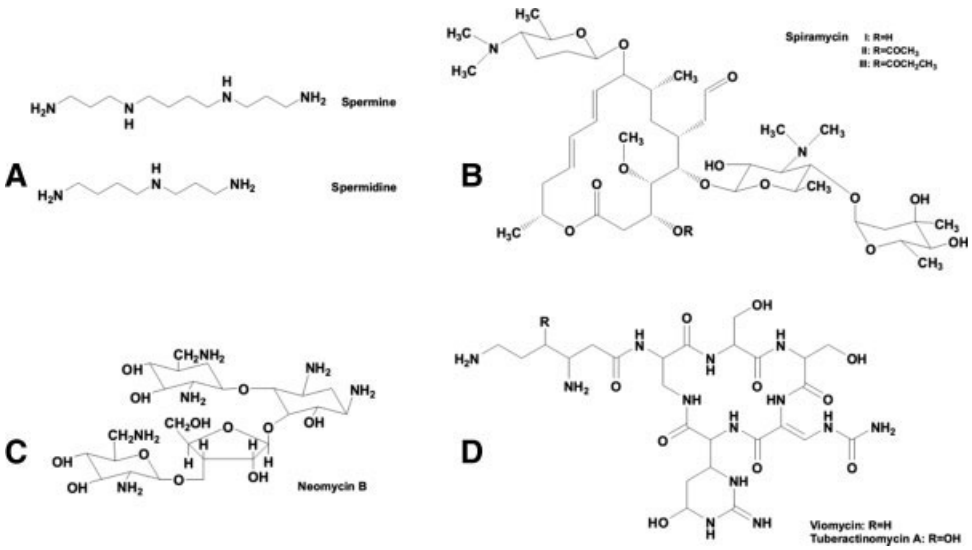

Figure 1. Chemical structures of four representative compounds that exert modulating effects on various ribozymes. (A) Spermine and spermidine; (B) Spiramycin; (C) Neomycin B; (D) Viomycin and tuberactinomycin A.

may provide the active centre with an amino group bearing a near neutral pK_a that serves as a stabiliser of intermediates or as a general acid/base during peptide-bond formation (*26*). Moreover, it was indicated that binding of spermine to 5S rRNA influences the PTase activity, a fact that reveals a regulatory role of this rRNA in the peptide-bond formation (*27*).

Sites other than the PTase centre that are bound by spermine in both 23S and 5S rRNA were found to be related with regions of the large ribosomal subunit implicated in functions that include positioning and translocation of tRNA substrates, binding of ribosomal factors and antibiotics and subunit association. In parallel, it was found that binding of spermine to 16S rRNA, although it affects several important functions, such as subunit association, binding of ribosome ligands and recognition of cognate tRNA fails to affect the catalytic properties of PTase (*28*).

Many different classes of important antibiotics such as puromycin, blasticidin S, sparsomycin, chloramphenicol, macrolides (Fig. 1B) and streptogramins target on 23S rRNA (*29*). Structural data, which are in excellent agreement with findings from earlier biochemical and genetic studies, have shown that the majority of them interact with the PTase hydrophobic crevice or with a second important hydrophobic crevice located in the entrance to the exit channel that nascent polypeptide travels through (*30*).

Puromycin, a structural analogue of aminoacyl-tRNA 3′-end, has played a central role in our understanding of the mechanism of peptide-bond formation (*31*). It binds to a 23S rRNA structural motif that encompasses several conserved residues implicated in the binding of the three termini of the acceptor and donor tRNAs (*32*). By binding to the ribosome and reacting with the peptidyl-tRNA, puromycin becomes incorporated into the nascent peptide chain, a fact leading to the termination of elongation. Blasticidin S, which structurally relates to puromycin and to aminoacyl-adenylyl terminus of aminoacyl-tRNA is a partial mimetic of P-site bound tRNA (*33, 34*). Sparsomycin, a slow-binding ligand of ribosome (*35, 36*) binds within the peptidyl transferase centre by stacking its uracil-like moiety to the universally conserved adenosine A2602 (*E. coli* numbering) of 23S rRNA. This induces substantial conformational alterations, which affects the correct positioning of both A-site and P-site tRNAs (*33*). It is interesting to note that sparsomycin triggers accurate translocation *in vitro* in the absence of EF-G and GTP. It has been proposed that sparsomycin-dependent rearrangement of A2602, coupled with stabilization of the peptidyl-tRNA interaction, leads to a decrease in the activation energy for translocation (*37*).

Chloramphenicol competes with the binding of tRNA 3′-end fragments to the A site and with puromycin. It was suggested that this drug inhibits the peptidyl transferase reaction by disturbing the binding of the 3′-CCA end of tRNA within the catalytic centre without weakening substrate binding. Structural studies of the archaeal (*H. marismortui*) and eubacterial (*Deinococcus radiodurans*) 50S subunits showed that chloramphenicol binds either to the hydrophobic crevice to the entrance of the peptide exit tunnel of *H. marismortui* (*33*) or to the PTase active site of *D. radiodurans* ribosomal subunits (*38*), suggesting that both of these sites may be physiologically relevant, and which most likely correspond to the two binding sites on eubacterial 50S subunits that have been inferred previously from binding (*39*) and kinetic studies (*40, 41*).

Macrolide antibiotics inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit in domain V of the 23S rRNA, within a hydrophobic pocket near the tunnel constriction (Fig. 2), thus blocking the passage of the nascent polypeptide through the exit tunnel a fact eventually leading to the premature dissociation of the peptidyl-tRNA during translation (*42*). A second target of macrolide antibiotics was recently found to be the assembly of 50S ribosomal subunit (*43*). 16-Membered macrolides, possessing disaccharide extensions at the C5 position of the lactone ring, can also inhibit peptide-bond formation (*34, 44*). Recent studies revealed that binding of macrolides to the ribosome is accomplished via a two-step mechanism; the first step, which is established, rapidly involves a low-affinity site located at the entrance of the exit tunnel, while the second step, mediated by slow conformational changes, pushes macrolides into a high-affinity site, deeper in the tunnel (*45*). Crystallographic studies of bacterial and archaeal 50S subunits revealed that contact sites for macrolide binding are the nucleotides A2058 and A2059 (*E. coli* numbering) of the 23S rRNA, which are located accessibly on the surface of the tunnel wall (*38, 46*). It has been reported that A to G mutation or posttranslational methylation of A2058 (*47*) as well as 2058G/2059G and 2058C/2059C dual mutations lend resistance to typical macrolides (*48*). This resistance can be overcome by the use of chemically modified macrolides, such as ketolides, which interact with other regions of the ribosomal tunnel (*49*).

Streptogramins, which are produced as a mixture of two chemically unrelated compounds, type A (unsaturated macrolactones) and type B (macrocyclic peptides of unusual amino acids), act synergistically on the ribosome and cause lethal bactericidal activity (*50*). Crystal structures of *D. radiodurans* 50S subunit complexed with a mixture of streptogramin A and B derivatives, the semisynthetic dalfopristin and quinupristin, respectively, have revealed that quinupristin interacts with the nucleotide A2058 (*E. coli* numbering) of 23S rRNA in the classical macrolide pocket, while dalfopristin binds directly within the peptidyl transferase centre affecting both A- and P-site occupation by the tRNA molecules. Also, these studies indicated that the synergistic effect derives from direct interaction of both compounds with a single nucleotide, A2062 (*51, 52*).

MODULATION OF RIBONUCLEASE P ACTIVITY

RNase P is a ubiquitous tRNA maturating enzyme that cleaves the 5′ leader sequence of precursor molecules. It harbours an essential RNA subunit (~130 kDa, Fig. 3), which is absolutely required for enzyme function: it is a true trans-acting ribozyme in Bacteria (*8*) and some Archaea (*9*), while in

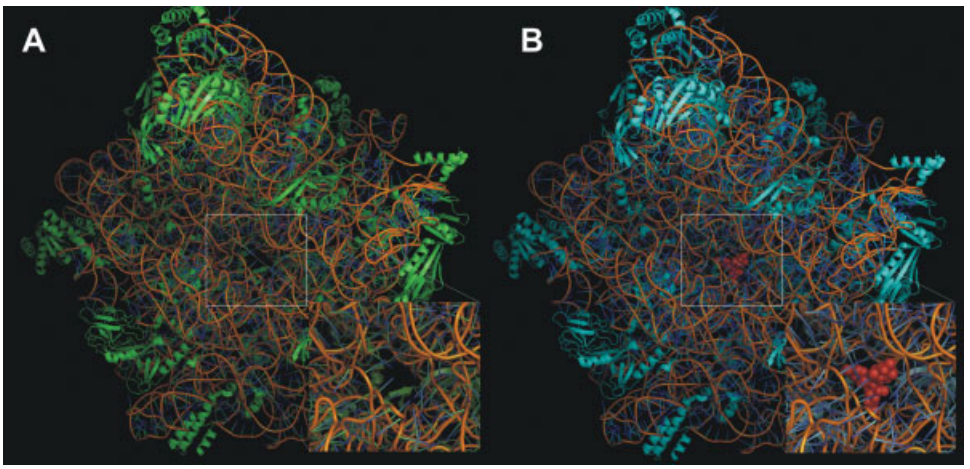

Figure 2. Crystal structure of the large ribosomal subunit from *H. marismortui*. A: In this view, the surface that interacts with the small ribosomal subunit is visible (PDB ID: 1JJ2; ref. (*11*)). The polypeptide exit tunnel can be seen in the centre. B: Same view of the ribosome, cocrystalised with the macrolide spiramycin (depicted as a space-filling model, coloured red) (PDB ID: 1KD1; ref. (*33*)). Insets: the central areas are depicted enlarged. All PDB files in this and following pictures were visualised using PyMol software (http://pymol.sourceforge.net/).

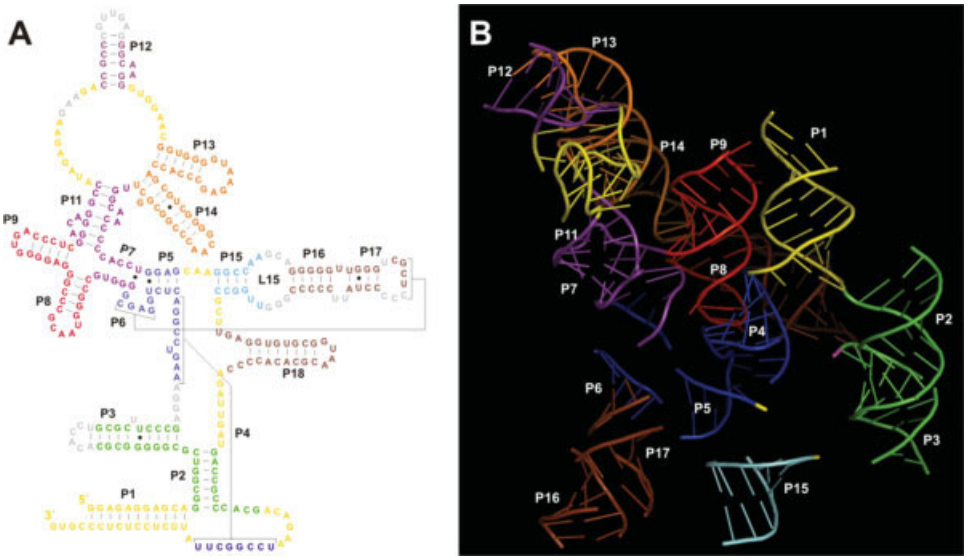

Figure 3. Secondary (A) and three-dimensional structure (B) of the type A RNase P RNA from *Thermotoga maritima* (PDB ID: 2A2E; ref. (*15*)). The secondary structure was acquired from RNase P Database (http://www.mbio.ncsu.edu/RnaseP/). Secondary structural elements were coloured as in (*53*). Residues coloured in gray are not modelled due to crystal disorder.

Eukaryotes, it retains traces of its catalytic abilities (*10*). In contrast to the RNA subunit, which displays striking similarities among all species, the protein complement differs significantly: bacterial RNase P RNA has one small (~14 kDa) protein partner (*53*), the archaeal ribonucleoprotein complex has four protein subunits, and the eukaryotic enzyme has up to 10 protein subunits, four of which are homologous to the archaeal ones. No archaeal or eukaryotic protein bears resemblance to the bacterial counterpart (*54*).

Mg^{2+} ions are absolutely required for RNase P function, assuming a dual role: they screen electrostatic repulsion between the negatively charged phosphates of the backbone so that it can fold correctly, and they participate in the reaction mechanism by activating the attacking nucleophile (H_2O) and stabilizing transition state intermediates (*55*). Until recently, divalent cations were thought to be the essential activators of all ribozyme reactions, but it has been proven that several small ribozymes can catalyse their reactions in the presence of mono-

valent cations alone, which are likely to play structural purposes (*56*).

Divalent Cations as RNase P Modulators

Alkaline earth cations Ca^{2+}, Sr^{2+} and Ba^{2+}, under appropriate conditions, can substitute to some extent for Mg^{2+} and exhibit a bimodal action at the kinetic phase of the *Dictyostelium discoideum* RNase P reaction (*57*). At low concentrations, these metals lower Mg^{2+} requirement, acting as nonessential–noncompetitive activators. At higher concentrations, they inhibit the RNase P reaction, acting as noncompetitive inhibitors. It is likely that this complexity is due to interactions of the divalent cations with two distinctive nonoverlapping sites on RNase P, one activating and one inhibitory. In another study, several divalent metal ions (Mg^{2+}, Mn^{2+}, Ca^{2+} and Sr^{2+}) have differential effects on cleavage site recognition and rescue of bacterial RNase P RNA enzymatic activity. It seems that efficient and correct cleavage is the result of co-operativity between divalent metal ions bound at different sites in the RNase P RNA-substrate complex (*58*). Mg^{2+} and Ca^{2+} are relatively abundant inside the cell and regulate a wealth of physiological functions. The above findings could be a reflection of *in vivo* regulation mechanisms of RNase P function or a remnant of the enzyme's primordial origin, but we currently have no data to support either speculation.

Protein Activation of a Ribozyme

RNase P RNA has drawn much of the scientific attention because of its enzymatic abilities, while the roles and functions of the protein complement have not been equally investigated. Nevertheless, it is well documented that the protein subunits exert an enhancement of substrate binding and catalysis by the RNase P ribozyme, which can be easily perceived as a modulating effect. The simplest model for such a study is the bacterial holoenzyme, because it comprises the RNA and only one small protein subunit. Nevertheless, its dissection proved anything but simple.

Careful kinetic analysis revealed that the presence of the protein does not have a drastic effect on the phosphodiester cleavage rate constant of the *B. subtilis* RNase P, but it increases affinity for Mg^{2+} cations that stabilise pre-tRNA binding (*55*). Substrate specificity is broadened as well, to other, non-tRNA substrates (*59*). It was also shown that the protein subunit contacts the 5′ leader sequence directly, thus further assisting the correct positioning of the pre-tRNA substrate at the active site (*60, 61*). However, the majority of the substrate binding surface lies in RNase P RNA. Moreover, the contribution of the protein to substrate binding appears to be more prominent for nonconsensus substrates (which lack common recognition elements), resulting in uniform binding of all pre-tRNA species (*62*). Thus, *E. coli* RNase P holoenzyme cleaves certain nonconsensus pre-tRNA substrates at a >900-fold higher k_{obs} under single turnover conditions in comparison with the RNA alone reaction.

It is now considered that the mechanism of protein activation of RNase P RNA is the stabilization of the native structural state of RNase P RNA upon association of the protein (*63*). This is achieved through subtle and local conformational changes rather than global ones (*64*). The activated ribozyme manifests its intrinsic properties, which include increased substrate affinity, enzyme dimer formation (*65*), *etc*., as described earlier. Nevertheless, there is no in-depth understanding of the phenomenon, due to literature inconsistencies and discrepancies between different species. Its clarification will require the construction of detailed structure models and the comparison of multiple versions of the enzyme *in vivo* and *in vitro*.

Small Molecule Modulators of RNase P Activity

This category includes mainly RNA-binding antibiotics, which are well-documented ribozyme inhibitors, but also other, less-studied molecules. Ribosome-targeting antibiotics, such as aminoglycosides, have also been found to inhibit RNase P activity (*66, 67*). It is well known that multivalent cations such as polyamines enhance the rates of a number of reactions involving polynucleotide substrates; spermidine is commonly added to RNase P RNA reactions. As we will describe, a different class of ribosomal inhibitors, macrolides (*68*), activate the 5′ maturation reaction of pre-tRNAs (*69*).

Aminoglycoside antibiotics are flexible, positively charged compounds that readily interact with various RNA molecules displaying a wide array of effects. When bound to the 16S rRNA, they induce a conformational change to the rRNA, which causes incorporation of noncognate aa-tRNAs and therefore the translational fidelity is lowered (*70*). On the other hand, neomycin B (Fig. 1C) and tobramycin, aminoglycosides that predominantly interact with other ribozymes (M1 RNA, hammerhead, hairpin, HDV, group I intron), inhibit catalytic activity by displacing essential Mg^{2+}. It is worth noticing that the inhibition is pH dependent, indicating that there is a direct relationship between the available positively charged amino groups in these antibiotics and their inhibitory potential (*68, 67*). Following that observation, Gopalan and coworkers rationally improved the aminoglycosides' inhibitory effect on bacterial RNase P by conjugation of several arginine residues (*71*) and, in a later work, guanidinium and lysyl groups (*72*). Moreover, the most potent of the three derivatives, NeoR5 (Neomycin B penta-argininyl conjugate) showed differing effectiveness on archaeal RNase P; type A RNase P (catalytically active RNase P RNA) was inhibited quite significantly, while type M RNase P (inactive RNase P RNA) was modestly activated (*72*). It was concluded that the inhibitor's potency depends on the molecular backbone as well as the length, flexibility and composition of the side chains. These data demonstrate that the use of rationally designed effectors on various systems has great analytical power for the study of mechanisms of action. It has been suggested that neomycin B binds to the P-15 loop (Fig. 3) of the *E. coli* RNase P RNA subunit (M1 RNA, Type A) in such a

way that it displaces a Mg^{2+} ion, which is probably involved in the chemistry of the cleavage (*67*). This result is partially corroborated by the effect of NeoR5 on archaeal RNase P activity (*72*). On the other hand, the NeoR5 derivative had the same inhibition effect on the *E. coli* RNase P reconstituted with the wild-type RNase P RNA or the P RNA mutant, which lacks the L15/P16/P17 elements (Fig. 3), suggesting that the explanation of the phenomenon might be more complex than it was initially proposed.

Apart from aminoglycosides, peptidyl transferase inhibitors such as puromycin, amicetin and blasticidin S, inhibit RNase P activity. Puromycin, a mimic of the 3′ terminal end of the aminoacyl-tRNA, was the first inhibitor of RNase P activity reported (*73*). Puromycin, amicetin and blasticidin S do not act as slow-binding inhibitors, as in the ribosome complex. Detailed kinetic analysis established their modes of inhibition as simple competitive in the case of puromycin and as noncompetitive for amicetin and blasticidin S. It was also shown that amicetin and blasticidin S do not have additive inhibitory effects, suggesting that these compounds compete for binding on a common site. Based on the comparison of Ki values of the three molecules, amicetin is the stronger inhibitor (*74, 75*).

Various studies have revealed the existence of additional compounds that inhibit RNase P activity such as porphyrins and porphines (*76*), which bind strongly the RNase P RNA and both precursor and mature tRNA, as well as retinoids (*77*) arotinoids (*78*), calcipotriol and anthralin (*79–81*), which function by a mechanism yet to be deciphered.

Ribozyme activators are rarely described, but one could notice that there have been no significant efforts in that direction. One of the main interests of our laboratory is to study the effects and to exploit any usefulness of chemical modulators of RNase P reaction. In a recently published study, we found that macrolides activate *E. coli* RNase P activity (both RNase P RNA-M1 RNA and holoenzyme), whereas there was no effect detected on eukaryal (*D. discoideum*) RNase P (*69*). Detailed kinetic analysis revealed that the macrolide antibiotic spiramycin (Fig. 1C) causes an 18- and 12-fold improvement in the activity status (k_{cat}/K_s) for the holoenzyme and M1 RNA reactions, respectively. Binding of spiramycin on the M1 RNA does not alter the ribozyme's dependence on Mg^{2+}, but, on the other hand, it causes a local structural rearrangement of the P10/11 element (Fig. 3), which is involved in substrate recognition. The element P10/11 has a conserved architecture in bacteria, which differs in eukaryotes, thereby providing a possible explanation for the insensitivity of *D. discoideum* RNase P to the stimulatory effect of macrolides. It was evidenced by a separate approach that the M1 RNA-spiramycin complex has increased affinity for the pre-tRNA substrate, also suggested by kinetic analysis.

Recently, the crystal structure of the RNase P RNA subunits from *Thermotoga maritima* and *Bacillus stearothermophilus* (*82, 83*) has been solved. The crystallographic data provide a structural model upon which earlier biochemical data can be integrated. Nevertheless, exact description of the modulation mechanisms of the aforementioned effectors will be available only after the resolution of the crystal structure of RNase P in complex with these compounds.

MODULATION OF GROUP I INTRON ACTIVITY

Group I introns are catalytic RNA molecules capable of self-splicing from primary transcripts in the absence of any protein factors or energy source *in vitro*. Self-splicing is realised in two steps and involves two consecutive transesterification reactions, which occur using a GTP molecule as cofactor. The cleavage reaction results in the ligation of the exons and the release of the linear intron (*84*).

Monovalent and divalent cations are necessary for the proper folding of group I introns and for catalysis *in vitro*, although the mechanism that these ions interact with the ribozyme is still unknown. There is a short region of the backbone within P4–P6 domain (Fig. 4) that has been called the 'metal ion core', and it is stabilised by the presence of several Mg^{2+} ions (*86*). It has been proposed that there are two site-specific metal ion-binding sites in the P4–P6 domain of *Tetrahymena* group I intron ribozyme. The metal ion core of this highly structured RNA seems to bind two divalent metal ions (*87, 88*). Moreover, P4–P6 domain has the ability to fold in high concentrations of monovalent

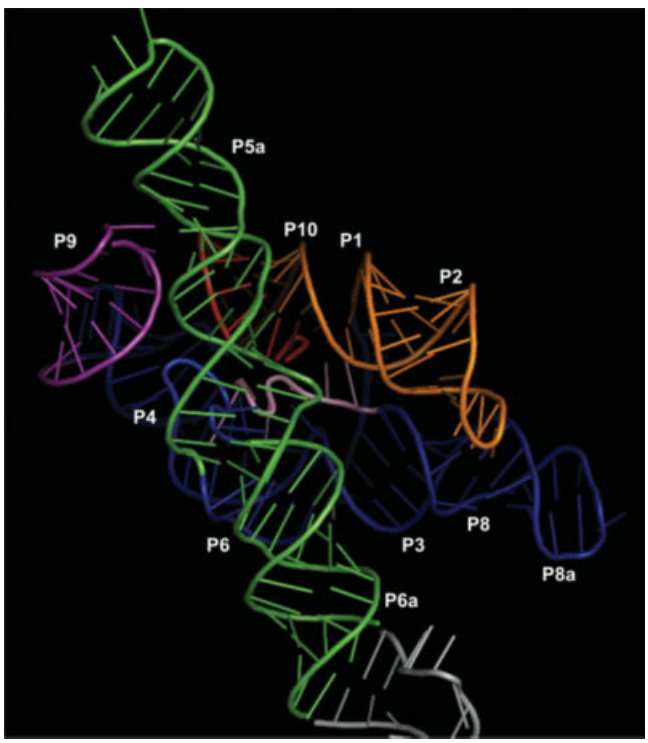

Figure 4. Three-dimensional structure (PDB ID: 1U6B, ref. (*85*)) of a group I intron. Elements of secondary structure are coloured according to (*85*). In the foreground, element P4-P6 is displayed coloured in green.

cations (Na^+), forming all of its tertiary structure except the central metal ion core (*88*), while there are only a few of divalent cations (Mg^{2+}, Mn^{2+},Ca^{2+} and Ni^{2+}) that facilitate this folding (*87*). It was recently shown that for the organization of the functional architecture of the *Tetrahymena* group I intron active site, a number of interactions, mediated by a peripheral metal ion, are important (*89*). This metal ion can affect differentially several steps of the catalytic process, indicating that its role is not precisely described as structural or catalytic.

The self-splicing reaction is inhibited by a number of small molecules of different structural types. The guanosine analogues (deoxy- and dideoxyguanosine) (*90*), the amino acid arginine (*91*), the antibiotic streptomycin (*92*), and the tuberactinomycin peptides viomycin (Fig. 1D) and di-β-lysylcapreomycin (*93*) are competitive inhibitors of group I intron self-splicing reaction. All these molecules contain guanidino residues in their structure that compete with the cofactor guanine for the G-binding site (*91*). The effect of all competitive inhibitors is dependent on guanosine and Mg^{2+} concentrations, thereby confirming the involvement of the G-binding site in the antibiotic/intron interaction and suggesting that electrostatic interactions may play a role in this association. Tuberactinomycin peptides seem to be much stronger inhibitors than other competitive inhibitors known so far, as they inhibit the splicing of group I intron in a range from 10 to 50 μM, in contrast to the other competitive inhibitors that accomplish inhibition at millimolar concentrations. It is worth mentioning that the pseudodisaccharide lysinomycin, which does not contain a guanidino group, is also a competitive inhibitor (*94*).

The linear intron, which results by self-splicing reaction, remains active and has the ability to perform an intramolecular transesterification resulting in a circular molecule. Interestingly, the introns can also act intermolecularly in the presence of subinhibitory concentrations of tuberactinomycin, leading to the formation of linear head-to-tail introns oligomers (*95*). Thus, the antibiotic stimulates the intron to react in trans instead of in cis. The tuberactinomycines are further able to induce the interaction of heterologous introns to form noncovalent complexes in the presence of the antibiotic (*95*). Polyamines have also been shown to stimulate group I intron splicing (*96*). Finally, a mitochondrial tyrosyl-tRNA synthetase has been shown to promote the formation of the catalytically active structure of the intron RNA (*97*).

The aminoglycoside antibiotics neomycin, gentamicin and tobramycin (*98*), tetracycline and pentamidine (*99*) and spectinomycin (*100, 101*) inhibit self-splicing in a noncompetitive manner. Aminoglycoside antibiotics seem to be the most potent inhibitors of self-splicing *in vitro* at concentrations as low as 10 μM. Interestingly, their function is independent of guanosine concentration (*102*). Aminoglycoside antibiotics also interact with the conserved functional core of group I introns, supporting the notion that they interfere directly with functional RNA elements (*103*). Their binding site could be near the catalytic core, although not necessarily at the G-binding site (*102*). It has been suggested, but not proven yet, that group I introns' and the ribosomal-decoding centre's susceptibility to aminoglycosides may imply that these two RNA modules adopt similar mechanisms of action (*104, 105*).

Despite the inhibitory effect of aminoglycosides, neomycin B seems to present an additional effect on group I intron splicing reaction. It strongly stimulates group I intron splicing *in vitro* at micromolar concentration (50–100 μM) by acting as Mg^{2+} mimic (*96*). The stimulation effect of aminoglycosides is observed at 10 mM Mg^{2+} concentration. No self-splicing is occurred in the absence of the neomycin in that concentration.

Recently, it has been demonstrated that another class of molecules, the coenzymes, are capable of inhibiting the group I self-splicing reaction. Coenzyme flavin mononucleotide (FMN), which does not contain a guanidino group, inhibits group I splicing in a competitive manner (*106*). In contrast, NAD^+ (*107*) and $NADP^+$ (*108*) act as noncompetitive inhibitors, which depend on guanosine and Mg^{2+} concentrations. Comparison of NAD^+ and $NADP^+$ inhibition suggests that the additional phosphate group of $NADP^+$ may interfere with either the binding or the catalytic action of Mg^{2+} ion, thereby increasing the inhibitory effect of $NADP^+$ over NAD^+. Other coenzymes that have displayed similar inhibitory action include thiamine pyrophosphate (*109*) and coenzyme A (*108*), which are competitive inhibitors, and pyridoxal phosphate (*110*), which is a noncompetitive inhibitor.

MODULATION OF HAMMERHEAD RIBOZYME ACTIVITY

The hammerhead ribozyme is a small catalytic RNA motif that is naturally found in several plant pathogenic viruses and viroids (*111, 112*), in which it facilitates the rolling cycle replication of their genomes. It is also found in several eukaryotes (*i.e.*, newt), which is involved in self cleavage of satellite DNA transcripts (*113*). This ribozyme has been engineered such that it can act in trans against other target RNA molecules and catalyse the cleavage of a specific phosphodiester bond and the production of 2′, 3′-cyclic phosphate and 5′-hydroxyl termini (*114, 115*). The hammerhead consists of three helical stems of arbitrary sequence and length, which intersect at an 11-nucleotide highly conserved catalytic core. Recent crystallographic data displayed the importance of interactions of peripheral elements present in native hammerhead motifs for the structural organization of the active site (Figs. 5A and 5B) (*116*). More importantly, it provided the basis for reconciling inconsistencies between previous biochemical data and the structure of the minimal hammerhead motif (Fig. 5C).

It was initially believed that the hammerhead ribozyme adopts its biological active tertiary structure only in the presence of divalent metal ions, such as magnesium [Mg^{2+}] and manganese [Mn^{2+}] (*117–119*). The best-characterised metal ion-binding site in the hammerhead ribozyme is at residues A9/G10.1, while other metal ion-binding sites are located around the G5 and A13 phosphate, near the cleavage site (*120*) (Fig. 5).

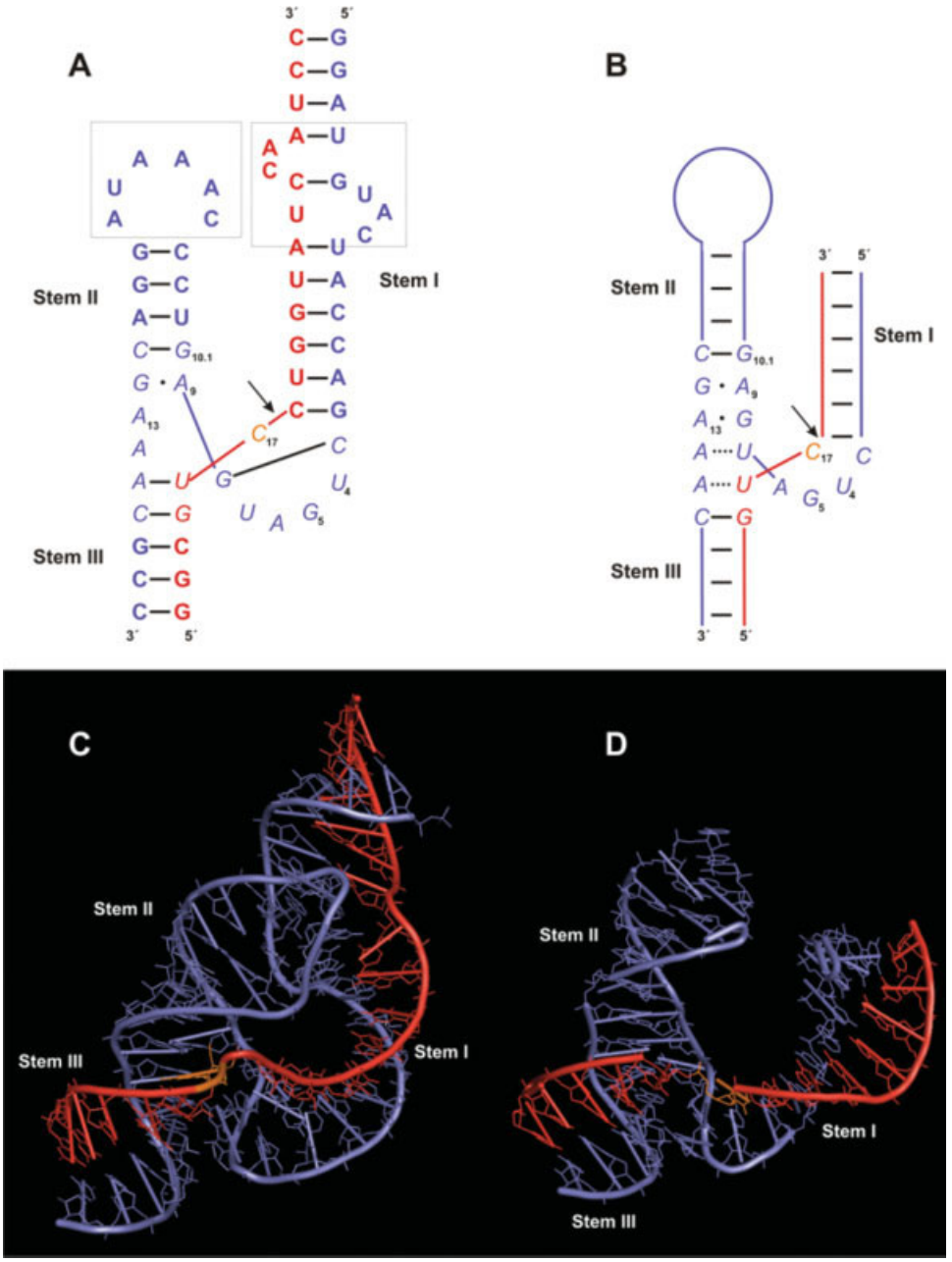

Figure 5. The hammerhead ribozyme. Secondary structure of the native (*Schistosoma mansoni*) (A) and minimal hammerhead motif (B). Substrate strand is coloured in red. Conserved residues are in *italics*. Black lines and black dots represent Watson-Crick and non-Watson-Crick base pairing, respectively. Coloured lines represent phosphate backbone continuity. The residue positioned directly 5′ to the cleavage site (C17) is coloured in orange. The arrow marks the cleaved bond. The dashed line frames enclose peripheral structural elements. C and D: Crystal structures of the same molecules (PDB IDs: 2GOZ and 1HMH; refs. (*116*) and (*14*)). Substrate strands are coloured red. The residue 5′ to the cleavage site is coloured orange. Characteristically, both molecules adopt similar folds, despite the absence of the peripheral elements from the minimal motif.

However, further studies have shown that high concentrations of monovalent ions, including sodium (Na^+), ammonium (NH^{4+}) and lithium (Li^+) not only can promote the adoption of the catalytically active fold but also can activate the ribozyme (*121, 122*). For example, although the cleavage of the hammerhead proceeds more efficiently in divalent cations, the reaction in 4 M LiCl is just 10-fold slower than that under standard conditions of 10 mM $MgCl_2$ (*56*). It was concluded that cations

simply serve for the screening of the electrostatic repulsion of the ribozyme's backbone, so that it can fold properly. Apart from facilitating the active fold, the divalent metal ion may contribute modestly to catalytic rate enhancement, probably through stabilization of transition state intermediates. Nevertheless, although suspected, the ribozymes' functional groups that constitute the catalytic centre remain to be uncovered (*121*).

Cations as Activity Modulators

The cleavage site of the wild-type substrate is located after the nucleotide C17. However, Borda and coworkers have described two other specific cleavage events that occur sequentially, the first between nucleotides G8 and A9 and the second between nucleotides C3 and U4 (Fig. 5B) of the minimal hammerhead motif HH16 (*123, 124*). Both of them occur when zinc ions are bound to the A9/G10.1 metal-ion-binding site. Moreover, the A9 cleavage can also be catalysed by other divalent metal ions, such as Pb^{2+} and Ni^{2+}, whereas the U4 cleavage is observed only in the presence of Zn^{2+}.

The U4 cleavage has two characteristic features: it is highly pH dependent, and it happens before the products of A9 cleavage are released. In particular, the products are generated only at a pH range from 7.7 to 8.5, in contrast to the hammerhead's wild-type substrate and A9 cleavage reactions, which take place from pH 6 to 8.3 (*118*). These two properties indicate that the U4 cleavage occurs under a specific conformation of the hammerhead ribozyme, which presumably requires specific ionic environment (*125*).

Antibiotics

Several groups of antibiotics have been found to interact with the hammerhead ribozyme. It has been observed that aminoglycoside antibiotics inhibit the cleavage reaction of the ribozyme (*126*). Among various aminoglycosides that have been tested (such as gentamycin, kanamycin, neomycin, paramomycin, lividomycin, ribostamycin, neamine, butirosin, apramycin and streptomycin), neomycin and 5-episisomicin have been reported as the strongest inhibitors (*127, 128*). Neomycin (Fig. 1C) inhibits the chemical cleavage step by stabilizing the ground state of the ES complex and destabilizing the transition state. The inhibitor's efficacy depends on the number of the positively charged groups and their basicity in physiologically relevant conditions. Evidently, inhibition arises when ammonium groups occupy metal-binding pockets on the ribozyme (*129*).

Generally, the backbone structure of the effector plays an important role in specific binding to the hammerhead ribozyme, because other polycations, like the polyamine spermine, do not inhibit catalysis at comparable concentrations (*130*). Additionally, spermidine, another polyamine, can reverse the Na^{+} inhibition, when added simultaneously with Mg^{2+}, by enhancing the cleavage rate of a hammerhead ribozyme (*131*).

Moreover, tetracyclines have been tested for interactions with the hammerhead ribozyme (*132*), and it has been revealed that chlorotetracycline and to a lesser extend tetracycline inhibit ribozyme activity. Like neomycin, chlorotetracycline's inhibition effect is overcome by high Mg^{2+} concentrations. In the case of neomycin, this phenomenon is well understood, but it is not adequately explained for chlorotetracycline.

Another class of drugs that affect the hammerhead activity is the peptide antibiotics viomycin, tuberactinomycin A (TubA) (Fig. 1D), tuberactinomycin B (TubB), enviomycin (TubN) and capreomycin. Studies, based on fluorescence technology, have shown that the cyclic pentapeptide compounds TubA and TubB are very strong hammerhead inhibitors (*127*). This inhibitory effect might be mediated by an OH group that all these inhibitors contain in α-position of the guanidino group in their six-membered ring. In the same study, a weaker but still important inhibitory effect was observed by a representative of type II polyketides, the antibiotic adriamycin.

Other Strategies

The engineered trans acting hammerhead ribozyme binds its substrate by base pairing using the sequences in its helices I and III. The stability of the complex and the catalytic rate of the reaction depend on the length and the base composition of the stems sequences. Thus, when the hammerhead stems are longer, the binding efficiency of the ribozyme is improved and the highly structured target RNAs are better cleaved. However, these cleavage products dissociate slower compared to those that are derived from ribozymes with shorter stems. In an effort to improve the efficiency of short-stemmed hammerhead motifs, oligonucleotides (9–12 residues) that bind on the substrate sequence 3′- or 5′-adjacent to the ribozyme were used (*133, 134*). In general, substrate association is increased by 5′ and 3′ facilitators due to a decreased dissociation constant of the ribozyme-substrate complex. 3′ end facilitators can also improve the cleavage rate (increase k_2) and product release (k_{cat}) as well. It should be noted that the resulting effects of 5′ and 3′ facilitators on the hammerhead reaction are highly dependent on the sequence composition of helices I and III, and the reaction conditions, particularly, ribozyme and substrate concentrations.

In a different approach, it has been noticed that a nonspecific RNA-binding protein derived from the p7 nucleocapsid protein (NC) of HIV type I enhances the cleavage of the hammerhead by increasing the k_{cat}/K_m and k_{cat} of the ribozyme (*135, 136*). This protein can also enhance the ability of the ribozyme to distinguish cognate RNA oligonucleotides (*137*). Another peptide from the C-terminal domain of the A1 hnRNP protein, called A1 CTD, can also increase the hammerhead activity, compared to other nonspecific-binding proteins, which do not influence the ribozyme catalysis (*135, 138*). In the above studies, the NC and A1 CTD proteins are considered to act like chaperons by preventing the ribozyme's misfolding.

Under standard conditions, the hammerhead ribozyme act as a ribonuclease, but it can also catalyse, even though less efficiently, the reverse reaction where the two-product RNAs are

ligated to reform the phosphodiester bond. However, when a disulfide crosslink was introduced in the hammerhead, the ligation rate of the ribozyme was significantly increased (*139*). This covalent crosslink was introduced five base pairs away from the catalytic core of the hammerhead and caused a reduction in the fluctuations between helices I and II resulting in a stimulation of the ligation reaction. Thus, this change in the molecule's thermodynamic properties led to a 100-fold increase of the ligation rate.

MODULATION OF THE HAIRPIN RIBOZYME

The hairpin ribozyme was discovered in the negative strand of the tobacco ring spot virus satellite RNA (-sTRSV) (*140, 141*), and is the second smallest, naturally occurring, self-cleaving RNA. Nevertheless, it also catalyses the ligation reaction, generating a circularised RNA species, which leads to an equilibrium between cleavage and ligation (reviewed in *142*).

The catalytic RNA/substrate RNA complex forms a two-dimensional hairpin structure having two helical domains (A and B) each containing two helical stems separated by an internal loop (Fig. 6). In the native hairpin motif, the two domains are connected via a four way junction, which is central for ribozyme activity, as by changing its conformation, the rate of cleavage can be modulated. Between the substrate and the ribozyme, two helices are formed, which allow the specificity of binding. Located between these two helices in the substrate are an N$^{\downarrow}$GUC sequence, where GUC is a required sequence, and the arrow marks the cleavage site (*144*). In the engineered minimum hairpin motif, the connecting junction is minimised, forming a hinge (two-way junction) (Fig. 6C). The catalytically active conformation is achieved through antiparallel docking of the two domains by induced fit. This state is characterised by a substantial rearrangement of the hydrogen bonding between nucleotide residues. Also an intricate network of coupled molecular motions connects distal parts of the molecule with its functional core (*145*). Folding of the natural four-way junction hairpin requires only micromolar magnesium ion concentration, whereas the minimal form requires three orders of magnitude higher of Mg^{2+} concentration for that purpose (*146*).

As described in detail for the hammerhead ribozyme, metal ions do not participate directly in the hairpin-cleavage mechanism and instead appear to play structural roles, such as in docking and adoption of active tertiary fold. Folding and docking can be promoted by a variety of ions, divalent (millimolar of magnesium, calcium and strontium) and monovalent (molar concentrations of sodium, lithium and ammonium) apparently with ranging efficiencies (*56, 147, 148*). Cobalt hexamine can substitute Mg^{2+} in both roles with comparable efficiency (*149–151*). This implies that the bivalent cations play exclusively structural roles in the form of hydrates. Consistent with this, the

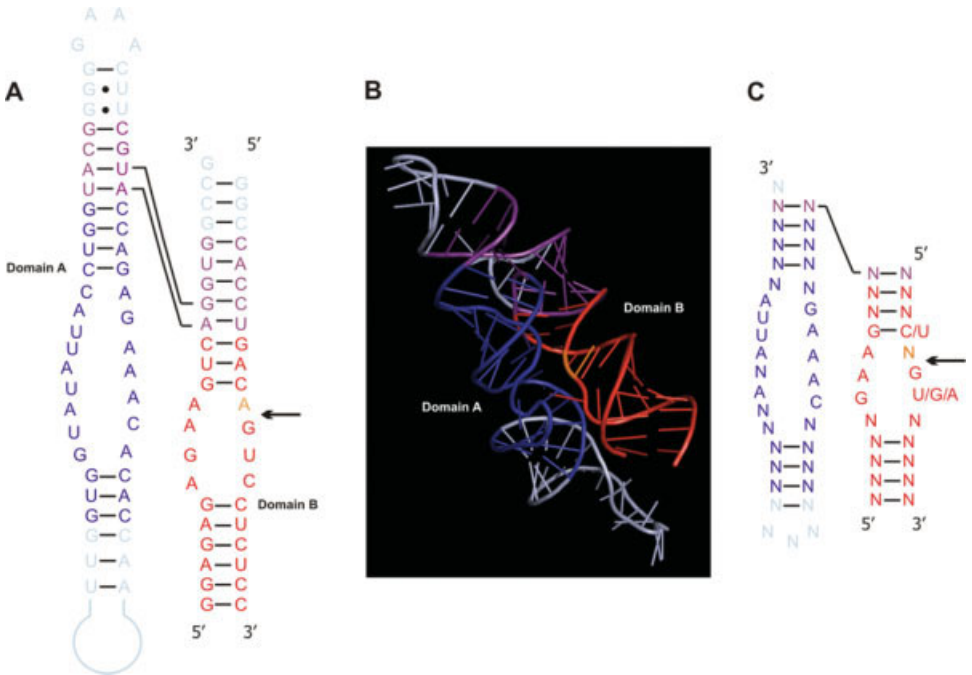

Figure 6. Secondary (A) and three-dimensional structure (B) (PDB ID: 1M5K, ref. (*143*)) of a native hairpin motif, adapted from (*13*). Domains A and B are coloured blue and red, respectively. Residues in purple comprise the four-way junction. The residue 5′ to the cleavage site is coloured orange. The arrow marks the cleavage site. C: Secondary structure of a minimal hairpin motif. The four-way junction is minimised to a hinge. The structural elements are coloured as earlier. 'N' represents any nucleotide, presumed that base pairing is conserved.

crystal structure of the ribozyme-inhibitor complex shows no metal ions in the active site (*143*).

It has also been shown that ionic requirements for ligation are similar to those for cleavage (*147*). These observations support a model in which ligation occurs via a simple reversal of the RNA-cleavage mechanism. The stability of the tertiary fold determines whether a hairpin motif is likely to perform ligation or cleavage. Ribozymes with a four-way helical junction bind 3′ cleavage products with much higher affinity than minimal hairpin ribozymes, and, in that case, the balance between ligation and cleavage shifts in favour of ligation (*152*). This is an illustrative example of ribozyme modulation through engineering of its structure.

Polyamines

The polyamine spermidine (Fig. 1A) was the first nonmetallic ionic entity shown to stimulate somewhat the hairpin-cleavage reaction in the absence of divalent cations (*147*). Tetraamine spermine (Fig. 1A) also supports a very efficient hairpin-cleavage reaction, almost as fast as magnesium ions in the same range of concentrations. In the presence of limiting magnesium concentrations, both polycations stimulate hairpin cleavage, with spermidine being much less effective than spermine (*153*). In another study, it was shown that spermine in combination with near physiological Mg^{2+} concentrations can actually enhance the activity of certain engineered motifs (*154*). Similarly to previously described strategies, certain spermine-amino acid conjugates stimulated hairpin cleavage at low magnesium ion concentration (*155*).

Antibiotics

Aminoglycoside antibiotics can cause inhibition of hairpin cleavage when added to a magnesium ion-assisted reaction. The degree of inhibition is correlated with the apparent binding constant of the antibiotic, the tighter the binding, the greater the degree of inhibition. 5-Epi-sisomicin is the most potent inhibitor, but the neomycin B also has significant inhibition properties, albeit not as significant as on hammerhead or RNase P ribozymes. All other representatives such as tobramycin, kanamycin B and gentamycin were much poorer inhibitors (*153*). In addition, aminoglycoside antibiotics stimulate hairpin ribozyme cleavage in the absence of metal ions with the best being apramycin, neomycin B and kanamycin B (*153*). As already described for other ribozymes, the inhibition is the result of essential Mg^{2+} displacement, while the ribozyme is activated when a polycationic compound is bound through positive groups to activating metal sites.

EPILOGUE

We have reviewed herein several cases where we observe extensive versatility in the divalent or/and monovalent ion dependence for the achievement of ribozyme functional folding and activation of catalysis. Moreover, small molecule effectors can characteristically inhibit and/or activate catalytic RNAs. Ligand-responsive ribozymes can play the roles of effective cis-regulators of gene expression. The mRNA that encodes *glmS* in Gram-positive bacteria contains a ribozyme that is activated by glucosamine-6-phosphate (GlcN6P), which is the metabolic product of the GlmS enzyme (*156*). The ribozyme cleaves the *glmS* transcript in response to rising concentrations of the metabolite, efficiently regulating its expression. Following nature's example, Yen et al. used a hammerhead motif as a cis-regulator of a reporter gene in mammalian cells. The ribozyme in the absence of a ligand is self cleaved, thus repressing the expression of the *lacZ* mRNA. Self cleavage was blocked by an inhibitor, which was identified by a high-throughput screen, resulting in the effective expression of the *lacZ* gene (*157*). We have recently shown that macrolides can significantly activate bacterial RNase P (*69*). This suggests that macrolides could also be used to regulate M1GS therapeutic implementations. It should be noted here that the ribozyme's native repertoire of ligands can be greatly expanded by fusion with an engineered aptamer, thereby constructing an allosteric ribozyme. Such biosensor modules can respond to a wide variety of signals (peptides, small organic molecules, oligonucleotides, metal ions and pH), and as a result, the allosteric ribozymes have equally wide variety of uses (*158–160*).

Similarly, a ribozyme can be 'switched' on and off by a protein partner. In that case, the folding of the ribozyme is allosterically regulated in the presence of the protein (ref. (*161*) and references therein).

Peptides and other small molecules could have similarly modulated ancestral RNAs, thus playing a crucial role into the evolutionary scenario of the RNA world (*7, 162, 163*). There has been a significant effort for the artificial recreation of a system of self-replicating catalytic RNA molecules, which would strengthen the RNA world hypothesis. The study of small molecule effectors can always provide this ongoing feat with answers. For example, it was recently shown that, apart from mutations in the context of *in vitro* evolution, the catalytic efficiency of RNA-dependent polymerases can also improve by hydrophobic anchors, which provide an organizing scaffold (164).

The above-selected examples demonstrate that ribozymes have a 'Swiss army knife' character, which has provided them with selective advantages throughout molecular evolution. This must be the reason why ribozymes, and other functional RNAs, in general, still assume critical functions in the biology of contemporary cells. The results of the structural analyses of the existing interactions between RNA and its molecular partners, together with the study of new binding motifs developed by *in vitro* evolution are being integrated into strategies for the construction and improvement of valuable, ribozyme-based, research and clinical tools.

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