Title

Ribozymes: structure and mechanism in RNA catalysis.

Authors

Scott, WG

Klug, A

Publication Date

2023-12-10

Peer reviewed

Ribozymes: structure and mechanism in RNA catalysis

William G. Scott and Aaron Klug

The hammerhead RNA is a small catalytic RNA found in a number of RNA virus genomes and virus-like RNAs. The recently determined crystal structures of hammerhead ribozymes reveal how a small RNA motif can fold up into a conformation suitable for mediating RNA cleavage.

A NEW FIELD in enzymology has emerged in the past decade with the discovery that RNA can act as an enzyme. First discovered in the cellular RNA-splicing and processing machinery in the form of self-splicing group I introns[1] and precursor tRNA-processing RNase P (Ref. 2), RNA catalytic activity in a number of smaller RNAs has subsequently been identified[3–5]. The small, naturally occurring catalytic RNAs are generally found in the genomes of RNA viruses and in virus-related RNAs, which are believed to replicate by a rolling-circle mechanism.

Recently, the crystal structure of one of these small catalytic RNAs, the hammerhead ribozyme, was elucidated by two research groups using different approaches. The first structure was that of the hammerhead ribozyme, in which the catalytic or 'enzyme' strand was composed of RNA and the RNA substrate was replaced with a 'substrate-analogue' strand composed of DNA. The DNA strand was employed as a competitive inhibitor to prevent catalytic cleavage[6]. The second hammerhead ribozyme structure was composed entirely of RNA with a single 2′-methoxyl modification at the active site to prevent cleavage[7]. Despite superficial differences, the largely conserved catalytic core region of both ribozyme structures is quite similar. The hammerhead ribozyme crystal structure, in conjunction with numerous experimental biochemical results, aids our understanding of RNA catalysis and its relation to RNA three-dimensional structure. Indeed, it has allowed us to propose a testable mechanism for RNA catalytic cleavage in the hammerhead ribozyme.

W. G. Scott and **A. Klug** are at the MRC Laboratory of Molecular Biology, Hills Road, Cambridge, UK CB2 2QH.

RNA enzymes and catalytic mechanisms

The group I intron catalyses the ligation of adjoining exons, using the 3′-OH of a guanine co-factor as a nucleophile to mediate *trans*-esterification, and RNase P (an RNA–protein complex whose RNA subunit possesses the catalytic activity of the enzyme) simply hydrolyses the phosphodiester backbone of precursor tRNA. Other RNAs, such as the group II intron[1], as well as several smaller self-cleaving RNAs, derived from RNA viruses and virus-like RNAs[3–5], also possess catalytic activity. The group II intron ligates adjoining exons using the 2′-OH of an adenosine within the intron to mediate *trans*-esterification, and in the process generates a 'lariat' product. Although the small, self-cleaving RNAs have very different conserved catalytic core sequences (and presumably, distinctly different three-dimensional structures), they have in common a metal-hydroxide-mediated *trans*-esterification that generates products having 5′-OH and 2′,3′-cyclic phosphate termini. Interestingly, $tRNA^{Phe}$, both in solution[8] and in the crystal form[9,10], has been observed to cleave catalytically and highly specifically in the presence of Pb^{2+}, yielding these same RNA-strand cleavage products.

The naturally occurring self-cleaving RNAs including $tRNA^{Phe}$ are single RNA molecules, but can be made into true enzymes exhibiting multiple substrate turnover simply by division into two strands of RNA. The nucleophiles implicated in the mechanisms of several catalytic RNAs are listed in Table I, together with the reaction products, which are characteristic of each type of ribozyme. A number of artificial ribozymes have now been produced by means of *in vitro* RNA selection methods[11]. These ribozymes are believed to employ other types of catalytic mechanisms.

Table I. Some characteristics of naturally occurring catalytic RNAs

Ribozyme species	Nucleophile	Reaction products
Group I intron	3′-OH of guanosine	5′ to 3′ joined exons and intron with 5′-guanosine and 3′-OH
RNase P	H_2O	5′-phosphate and 3′-OH
Group II intron	2′-OH of adenosine	5′ to 3′ joined exons and intron with 2′–3′ lariat joined at A and 3′-OH tail. Also acts as a DNA endonuclease when bound to a protein
Hammerhead ribozyme	divalent metal hydroxide, e.g. $[Mg(H_2O)_5(OH)]^+$	5′-OH and 2′,3′ cyclic phosphatase
Hairpin ribozyme	divalent metal hydroxide, e.g. $[Mg(H_2O)_5(OH)]^+$	5′-OH and 2′,3′ cyclic phosphatase
Hepatitis delta virus ribozyme	divalent metal hydroxide, e.g. $[Mg(H_2O)_5(OH)]^+$	5′-OH and 2′,3′ cyclic phosphatase
$tRNA^{Phe}$	divalent lead hydroxide, e.g. $[Pb(H_2O)_n(OH)]^+$	5′-OH and 2′,3′ cyclic phosphatase

The hammerhead RNA is small and well characterized

Because it is small and has a simple cleavage mechanism, the hammerhead ribozyme is perhaps the best experimentally characterized RNA enzyme, and therefore, is a clear candidate for structural studies. Owing to the dedicated efforts of a number of biochemists, a wealth of information regarding the conserved base requirements in the catalytic core of the hammerhead RNA, as well as the chemical nature of the divalent-metal-catalysed strand-cleavage reaction, has been made available[5,12,13].

The hammerhead motif consists of three base-paired stems flanking a central core of 15 conserved nucleotides. (Fig. 1). The conserved central bases are essential for ribozyme activity. Most of these conserved bases cannot form conventional Watson–Crick base pairs, but instead form more complex structures, which mediate RNA folding and catalysis. Substitution of any of the conserved bases with other naturally

PII: S0968-0004(96)10026-8

occurring bases[14], or sometimes even artificial alteration of their functional groups[5], results in diminished catalytic activity. In addition, two sets of base pairs in stem III and one pair in stem II are conserved; changing these to other base pairs either impairs or abolishes catalytic function. The crystal structure of the hammerhead ribozyme provides rationalizations for several sets of previous experimental observations[7], although a few other sets of results are at odds with the crystal structures (and sometimes contradict one another)[5].

The hammerhead RNA, like all other naturally occurring ribozymes, is a metalloenzyme[15] and requires a divalent metal ion, such as Mg^{2+}, to mediate catalytic cleavage. As with Pb^{2+}–$tRNA^{Phe}$, the divalent metal ion is thought to be hydrated, and becomes active when it binds to the RNA and ionizes, i.e. the active form is an RNA-bound metal hydroxide that acts by abstracting a proton from the 2′-OH at the cleavage site. The rate of divalent-metal-ion-assisted catalytic cleavage generally increases with decreasing pKa of the metal hydroxide (see Table II), strongly suggesting the active species is indeed a metal hydroxide[12,13]. However, exceptions to this rule (such as Pb^{2+}, which specifically cleaves $tRNA^{Phe}$ but not the hammerhead RNA) do exist, indicating that other factors, such as ionic radius and 'hardness' of the metal ion might also play a role in determining catalytic activity*.

Finally, replacement of the *pro*-R (but not the *pro*-S) phosphate oxygen (see Fig. 2) at the active site with a sulphur reduces hammerhead catalytic activity in the presence of Mg^{2+}; this activity can be rescued partially by the addition of softer (hence more thiophilic) divalent metal ions such as Mn^{2+} and Cd^{2+} (Ref. 18). This latter result indicates that Mg^{2+} (a relatively hard Lewis acid) binds directly to the *pro*-R oxygen at the cleavage site (see also Fig. 2b).

Crystal structure of the hammerhead ribozyme

Despite differences in nucleotide composition, phosphate backbone connectivity, crystallization conditions and crystal-packing interactions, the three-dimensional structure of the catalytic core of the all-RNA hammerhead ribozyme[7] is almost identical to that of the hammerhead ribozyme in complex with a DNA substrate inhibitor[6], suggesting that both structures represent the correct fold for an active hammerhead ribozyme in solution. Chemical crosslinking experiments also demonstrate that a hammerhead ribozyme, restrained to the crystal structure fold, has unaltered cleavage activity[19], further suggesting that the crystal structure indeed represents the correct fold of the hammerhead ribozyme.

The global conformation of the all-RNA hammerhead ribozyme is depicted in Fig. 3a as a roughly γ-shaped fold. Stem II and stem III are approximately co-axial, with stem I and the catalytic pocket branching away from this axis. Stem II, augmented by two GA, reversed-Hoogsteen base pairs and an unusual AU base pair, stacks directly upon stem III, forming one pseudo-continuous helix. The helix is not actually continuous, because it incorporates a three-strand junction where the active site cytosine (C-17) is squeezed out of the helix and forced into the four-nucleotide catalytic pocket, which is formed by a sharp turn in the hammerhead enzyme strand. This turn is identical in sequence and structure to the uridine turn found in the anticodon loop of $tRNA^{Phe}$ (Ref. 6). The phosphate backbone strands, which diverge at the three-strand junction, subsequently reunite to form stem I. These structural features are illustrated schematically in Fig. 3b, which is colour-coded to complement Fig. 3a.

Structural details and a proposed mechanism for RNA catalysis

The uridine turn in the hammerhead-RNA smoothly connects stem I to the augmented stem II helix by bending the enzyme strand of the ribozyme molecule, forming a highly structured pocket into which the cleavage base is positioned suggestively. The $tRNA^{Phe}$ uridine turn binds divalent metal ions

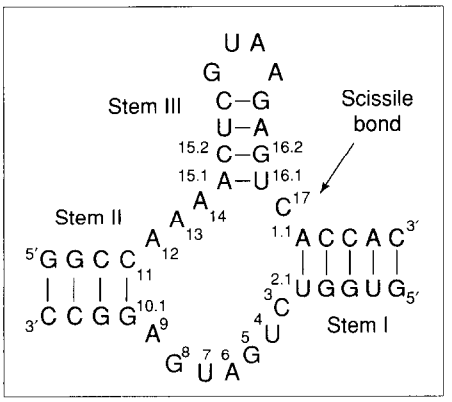

Figure 1

Hammerhead RNA secondary structure and cleavage site. The secondary structure of the all-RNA hammerhead ribozyme used for structural determination, consisting of a 16-nucleotide enzyme strand and a 25-nucleotide substrate strand. The conserved bases shown as red letters are required for catalytic activity. The cleavage site is indicated.

such as Mg^{2+} and Pb^{2+}, suggesting that the catalytic pocket in the hammerhead ribozyme is also capable of binding the catalytically active divalent metal ion. Difference Fourier analyses of the all-RNA hammerhead ribozyme crystals reveal a number of peaks of different electron density, which we have assigned as $Mg(H_2O)_6^{2+}$ complex ions, based on distance geometry criteria. Included is a single peak found near the catalytic pocket corresponding to a $Mg(H_2O)_6^{2+}$ complex ion, which can make hydrogen-bonding contacts with the exocyclic amines on C-3 in the catalytic pocket, and on C-17, the cleavage-site nucleotide. The cytosine, corresponding to C-3 in the hammerhead RNA, makes similar contacts with hydrated metal ions in the $tRNA^{Phe}$ uridine turn.

We have proposed a mechanism, based on the position of the $Mg(H_2O)_6^{2+}$ complex ion near the catalytic pocket of the hammerhead ribozyme, as well as on the similarly situated metal-binding sites in the uridine turn of $tRNA^{Phe}$, in which the $Mg(H_2O)_6^{2+}$ complex ion

Table II. Relative cleavage rates for hammerhead ribozyme with various divalent metals[a]

Metal	pKa[b]	Relative rate	Relative $[M^{2+}(OH)^-]$[c]	Hardness	Pauling's ionic radius
Ca^{2+}	12.9	1/16	1/32	Hard	0.99 Å
Mg^{2+}	11.4	1.0	1.0	Hard	0.65 Å
Mn^{2+}	10.6	~10	6.3	Soft	0.80 Å
Co^{2+}	10.2	~10	15.9	Borderline	0.72 Å
Cd^{2+}	9.6	~6–10	63.1	Soft	0.97 Å
Pb^{2+}	7.7	No cleavage	5013	Borderline	1.21 Å

[a]See Refs 12, 13, 16, 17 and 31.
[b]$pKa = pH - \log([A^-]/[AH])$.
[c]Relative metal hydroxide concentration as compared to $[Mg^{2+}(OH)^-]$ at pH7.0 free in solution.

*So-called 'hard' metal ions, such as Mg^{2+}, are Lewis acids that interact with 'hard' Lewis bases such as phosphate oxygens and H_2O in preference to 'softer' Lewis bases such as the exocyclic functional groups of nucleotide bases. 'Hard' interactions are predominantly electrostatic, whereas 'soft' interactions are dominated by orbital interactions[16,17].

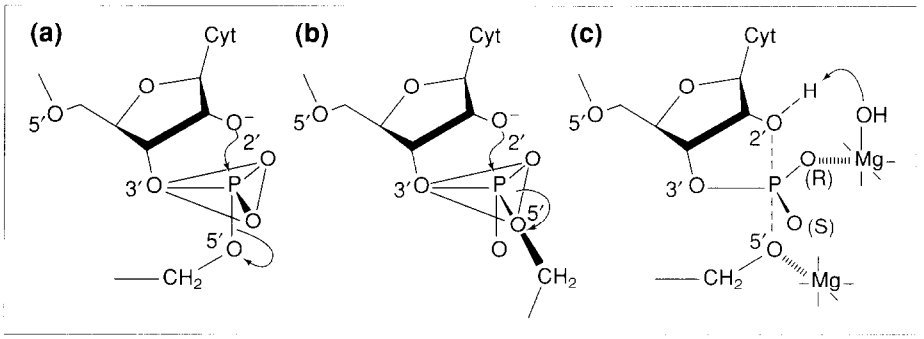

Figure 2

Chemistry of hammerhead cleavage mechanisms. Illustration showing **(a)** 'in-line' and **(b)** 'adjacent' mechanisms of phosphodiester-strand cleavage. **(c)** Two possible sites for magnesium-mediated RNA-strand cleavage. *Pro*-R and *pro*-S non-bridging phosphate oxygens are indicated. Note that one Mg^{2+} can fulfil both the roll of binding to the *pro*-R oxygen and of abstracting the 2′-proton by a metal-bound hydroxide, and that there is now experimental evidence[28] against the existence of a second metal ion acting as a Lewis acid by binding directly to the bridging 5′-leaving oxygen.

first 'docks' in the catalytic pocket by interacting with C-3 and C-17 as noted above (see Fig. 4a). Independent experimental corroboration for this initial interaction has recently emerged; removal of the exocyclic amine from either C-3 or C-17 causes the dissociation constant for the catalytic $Mg(H_2O)_6^{2+}$ to increase by almost one order of magnitude[19]. Although both hammerhead RNA crystal structures have C at position 17, this C can be replaced with A or U (A-17 works almost as well as C-17, but the activity for U-17 is somewhat reduced[11]). Essentially, the same 'docking' interaction could still take place with A, where the $Mg(H_2O)_6^{2+}$ complex ion now interacts with the exocyclic amines on A-17 and C-3, but the analogous interaction would be weaker in the case of U-17, which lacks an exocyclic amine, and thus could explain the observation that A replaces C at position 17 more effectively than does U.

We propose that the metal complex ion is then drawn in towards the cleavage site 2′-OH group until it is within striking distance[7]. (The trajectory and final position of the complex ion are both inferred from the metal positions in the uridine turn of $tRNA^{Phe}$.) As the metal is positioned, one of the six H_2O molecules bound to the metal ion is displaced by the *pro*-R phosphate oxygen at the cleavage site, and that direct coordination with this phosphate oxygen assists in orienting and perhaps in ionizing one of the remaining H_2O molecules, which is now close to the 2′-OH group, i.e. binding the phosphate oxygen might lower the effective pKa of the hydrated magnesium ion, thus activating it. Loss of a proton generates a nucleophilic metal hydroxide, which in turn acts by abstracting the 2′-OH proton from the ribose in the cleavage site, initiating nucleophilic attack at the phosphorus and formation of the penta-coordinated 2′,3′-cyclic phosphate transition state or intermediate shown in Fig. 4b.

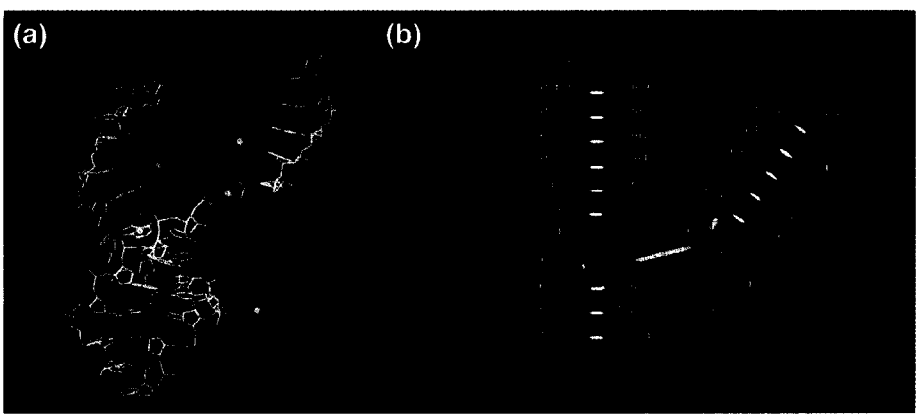

Figure 3

The crystal structure of the hammerhead ribozyme. **(a)** The three-dimensional structure of the all-RNA hammerhead ribozyme, showing the enzyme strand in red and the substrate strand in yellow. The cleavage-site base (C-17) is highlighted in green. Difference electron density interpreted as $Mg(H_2O)_6^{2+}$ sites is shown as purple peaks containing blue spheres corresponding to the complex ion center of mass. **(b)** A corresponding schematic diagram indicating the location of stems I, II and III, the catalytic pocket, the augmented stem II helix and the tetraloop. The colour-coding is preserved and the essential nucleotides are shown as shadow letters. The universal numbering scheme is indicated.

In-line or adjacent nucleophilic attack?

Both hammerhead crystal structures[6,7] reveal that their respective substrate analogues (e.g. DNA, and RNA with a 2′-O-methyl-cytosine, each incorporated to prevent cleavage in the crystal) are not in a conformation that would support an 'in-line' mechanism of RNA-strand cleavage[6]. This might be due to the absence of an unmodified 2′-OH at the cleavage site, but it is interesting to note that the same is true for $tRNA^{Phe}$ crystals with Pb^{2+} bound at the cleavage site, where the active 2′-OH is unprotected, except for being in a low-pH environment[9]. However, experimental evidence obtained by three research groups clearly demonstrates that the hammerhead-RNA cleavage reaction proceeds by an in-line mechanism[21–23] (see Fig. 2a). The reaction requires a change in the conformation of the phosphate backbone[6,7,9] and results in inversion of configuration of the reaction product. This was demonstrated using thio-substituted phosphate oxygens. These experiments also demonstrated that the catalytic metal interacts with the *pro*-R, but not the *pro*-S phosphate oxygens.

A simple 'adjacent' mechanism (like the one originally proposed for Pb^{2+}-bound $tRNA^{Phe}$ crystals[10]), which would not require a rearrangement of the phosphate backbone conformation, but which would require pseudo-rotation of a penta-coordinated phosphate intermediate[10,24], leading to retention of configuration in the product[24], is ruled out.

One metal ion or more?

There are three potential opportunities for catalysts to accelerate the hammerhead self-cleavage reaction. The first is for a base catalyst to abstract the proton at the cleavage site 2′-OH; this role appears to be fulfilled by a divalent metal hydroxide, as discussed above. The second is for this same metal ion, or possibly another, to polarize the phosphate by binding to the *pro*-R oxygen directly. The geometric constraints permit both roles to be fulfilled by a single metal ion, and the single metal mechanism possesses the added advantage of allowing the $Mg(H_2O)_6^{2+}$ to become

activated by lowering the effective pKa upon binding the *pro*-R oxygen, as noted above. Both of these well-established interactions are illustrated in Fig. 2b. The third, and more contentious, opportunity for metal catalysis is for an acid to stabilize the 5′-bridging-oxygen leaving group as the scissile bond breaks. This can, in principle, be accomplished either by protonation of the 5′-oxygen as negative charge begins to accumulate (general acid catalysis) or by direct coordination of the 5′-oxygen with a divalent metal ion such as Mg^{2+} (Lewis acid catalysis).

The latter mechanism has been proposed based on molecular orbital calculations of a model compound in the gas phase[25]. Evidence for the absence of a kinetic isotope effect in hammerhead-ribozyme phosphodiester cleavage has recently been obtained, indicating the non-existence of a proton-transfer process in the rate-limiting step of the cleavage reaction[26]. This result was interpreted to suggest that the 5′-oxygen is not protonated by a general acid catalyst, but rather is bound directly by a Lewis acid catalyst such as a second Mg^{2+} ion, as shown in Fig. 2b. However, any mechanism in which the rate-limiting step of the reaction does not involve proton abstraction or transfer is equally consistent with the data. For example, a mechanism in which rearrangement of the phosphate backbone into a conformation suitable for in-line attack is rate-limiting[6] should also show a lack of the kinetic isotope effect. Indeed, substitution of the leaving oxygen with a sulphur yields a hammerhead substrate whose leaving group now should be stabilized by soft divalent metal ions relative to the harder Mg^{2+}, if the proposed Lewis acid catalyst exists. However, unlike the case of the group I intron[27], no such reaction-rate acceleration was observed, suggesting that the leaving oxygen is not stabilized by a divalent metal ion binding directly to it[28]. These findings are likely to be generalized for the other small self-cleaving RNAs including $tRNA^{Phe}$. In the case of $tRNA^{Phe}$, as in the case of the hammerhead ribozyme, it is interesting to note that only one metal ion can be found at the cleavage site in the crystal structure.

Concluding remarks

The crystal structure of the hammerhead ribozyme, like that of $tRNA^{Phe}$ elucidated 21 years before[29,30], has revealed much information about RNA structure and function. In addition, the hammerhead RNA structure allows many new insights into how the three-dimensional structure mediates catalytic cleavage, including how the cleavage-site base is positioned in the uridine turn or catalytic pocket of the molecule, and how this pocket might bind and position the hydrated magnesium ion responsible for catalysing the first step of the hammerhead cleavage reaction. However, both hammerhead RNAs used for elucidating the crystal structure are essentially ribozymes bound to substrate-inhibitor analogues (either with a 2′-hydrogen or a 2′-methoxyl replacing the 2′-OH at the active site), and this, by necessity, gives only partial information about the cleavage-reaction mechanism. What remains to be elucidated, through the use of other modified bases and by time-resolved crystallographic techniques, is the structure of the reaction intermediate(s) complete with all catalytic metals bound unambiguously.

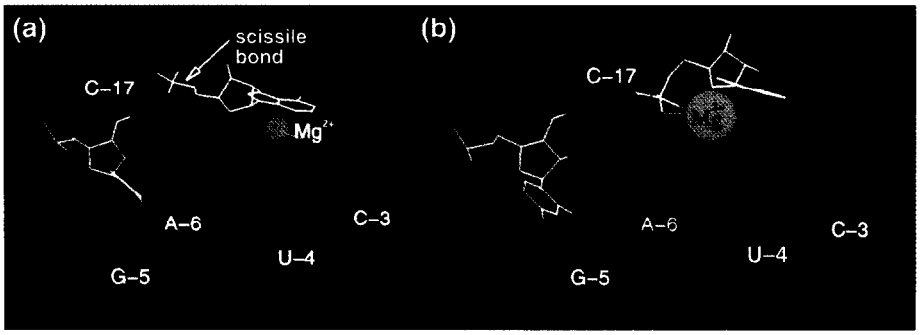

Figure 4

Metal binding and catalysis. **(a)** A potentially catalytic $Mg(H_2O)_6^{2+}$ site is located adjacent to the exocyclic amines of C-17 and C-3 in the hammerhead ribozyme catalytic pocket. **(b)** A possible structure of the hammerhead RNA transition-state. The implications of the experimental biochemical results for the mechanism of hammerhead RNA catalytic cleavage are: (1) that a hydrated metal binds directly to the *pro*-R phosphate oxygen at the cleavage site as (or possibly after) one of its chelated water molecules ionizes to form a metal hydroxide, and this nucleophile abstracts the labile proton from the 2′-OH of the cleavage-site base; (2) the reaction proceeds by an in-line, $S_N2(P)$ mechanism; and (3) the phosphate backbone must undergo a conformational change before or during cleavage to make in-line attack possible. Our proposed mechanism, based on the hammerhead RNA structure as well as comparisons with metal binding in the uridine turn of $tRNA^{Phe}$, adheres to these three conditions. Substitution of C-17 with adenosine or uridine at the active site maintains a functional ribozyme, although U-17 functions less well than A-17 and C-17 (Ref. 12). This fact is accounted for in both stages of the proposed mechanism. In the first step, the interaction with the exocyclic amine could still take place with A, but the analogous interaction would be somewhat weaker in the case of U-17, which lacks an exocyclic amine. In the second step, the base itself of the cleavage-site nucleotide stacks on A-6 in the catalytic pocket. Such a stabilization interaction may also take place with adenosine or uridine substituting for cytidine at the cleavage site.

Acknowledgements

We thank J. Finch, M. Gait, D. Brown, A. Kirby, S. Price, K. Nagai, G. Varani, B. Stoddard, K. Flaherty, D. McKay, O. Uhlenbeck and two anonymous referees for helpful advice. W. G. S. thanks the American Cancer Society for a postdoctoral fellowship (grant number PF-3970).

References

1 Cech, T. R. (1993) in *The RNA World* (Gesteland, R. F. and Atkins, J. F., eds), pp. 239–269, Cold Spring Harbor Press
2 Gopalan, V., Talbot, S. J. and Altman, S. (1994) in *RNA–Protein Interactions* (Nagai, K. and Mattaj, I. W., eds), pp. 103–126, IRL Press
3 Symons, R. H. (1992) *Annu. Rev. Biochem.* 61, 641–671
4 Symons, R. H. (1994) *Curr. Opin. Struct. Biol.* 4, 322–330
5 Tuschl, T., Thomson, J. B. and Eckstein, F. (1995) *Curr. Opin. Struct. Biol.* 5, 296–302
6 Pley, H. W., Flaherty, K. M. and McKay, D. B. (1994) *Nature* 372, 68–74
7 Scott, W. G., Finch, J. T. and Klug, A. (1995) *Cell* 81, 991–1002
8 Sampson, J. R. *et al.* (1987) *Cold Spring Harbor Symp. Quant. Biol.* 54, 267–275
9 Brown, R. S. *et al.* (1983) *Nature* 303, 543–546
10 Brown, R. S., Dewan, J. C. and Klug, A. (1985) *Biochemistry* 24, 4785–4801
11 Chapman, K. B. and Szostak, J. W. (1994) *Curr. Opin. Struct. Biol.* 4, 618–622
12 Dahm, S. C., Derrick, W. B. and Uhlenbeck, O. C. (1993) *Biochemistry* 32, 13040–13045
13 Pan, T., Long, D. M. and Uhlenbeck, O. C. (1993) in *The RNA World* (Gesteland, R. F. and Atkins, J. F., eds), pp. 271–302, Cold Spring Harbor Press
14 Ruffner, D. E., Stormo, G. D. and Uhlenbeck, O. C. (1990) *Biochemistry* 29, 10693–10702
15 Pyle, A. M. (1993) *Science* 261, 709–714
16 Pearson, R. G. (1963) *J. Am. Chem. Soc.* 85, 3533–3539
17 Fraústo da Silva, J. J. R. and Williams, R. J. P. (1993) in *The Biological Chemistry of the Elements*, pp. 34–36, Clarendon Press
18 Dahm, S. C. and Uhlenbeck, O. C. (1991) *Biochemistry* 30, 9464–9469
19 Sigurdsson, S. Th., Tuschl, T. and Eckstein, F. (1995) *RNA* 1, 575–583

20 Murray, J. B., Adams, C. J., Arnold, J. R. P. and Stockley, P. G. (1995) *Biochem. J.* 311, 487–494
21 van Tol, H. *et al.* (1990) *Nucleic Acids Res.* 18, 1971–1975
22 Slim, G. and Gait, M. J. (1991) *Nucleic Acids Res.* 19, 1183–1188
23 Koizumi, M. and Ohtsuka, E. (1991) *Biochemistry* 30, 5145–5150
24 Eckstein, F. (1985) *Annu. Rev. Biochem.* 54, 367–402
25 Taira, K. *et al.* (1990) *Protein Eng.* 3, 691–701
26 Sawata, S., Komiyama, M. and Taira, K. (1995) *J. Am. Chem. Soc.* 117, 2357–2358
27 Piccirilli, J. A. *et al.* (1993) *Nature* 361, 85–91
28 Kuimelis, R. G. and McLaughlin, L. W. (1995) *J. Am. Chem. Soc.* 117, 11019–11020
29 Robertus, J. D. *et al.* (1974) *Nature* 250, 546–551
30 Kim, S-H. *et al.* (1974) *Science* 185, 435–440
31 Burgess, J. (1978) *Metal Ions in Solution*, Ellis Horwood Ltd

Methods and reagents

Carriers for precipitating nucleic acids

Methods and reagents is a unique monthly column that highlights current discussions in the newsgroup bionet.molbio.methds-reagnts, available on the Internet. This month's column provides some tips for the precipitation of DNA and RNA samples. For details on how to partake in the newsgroup, see the accompanying box.

A recurring question on methds-reagnts concerns the best method of precipitating DNA or RNA when preparing them for enzymatic reactions. The most common technique for precipitation of DNA is with the addition of 0.1 vols of 3 M sodium acetate (pH5.5) and either 2–2.5 vols of ethanol or 0.8–1.0 vols of isopropanol. The mixture is placed at –70°C for 15 mins to several hours before being centrifuged at top speed in an eppendorf table top centrifuge for 10–15 mins at 4°C (Ref. 1).

Oh pellet, sweet pellet

Even though claims of 100% recovery are sometimes made, pessimistic netters feel this is over-estimated and in practice they typically expect to lose up to 50% of their DNA upon precipitation, especially if the DNA is less than 200 bp long or of low concentration. They therefore feel the necessity of doing everything possible to prevent such losses.

New WWW service from BIONET

The latest messages posted to the bionet as well as all past archived messages are located at net.bio.net and all you will need to do in order to read and/or post to any of the newsgroups is point your World Wide Web browser to the URL http://www.bio.net and then click on the 'Access the BIOSCI/ bionet Newsgroups' hyperlink.

A new hypermail archiving system now gives you the advantages of USENET without requiring a local news server. The message headers are threaded by default, but messages can also be displayed chronologically or sorted by author or subject line. This capability gives you, in effect, a threaded newsreader through the Web. If you have any questions or encounter any problems with the new server, please report them to biosci-help@net.bio.net

Although the small amount of DNA used for most molecular biology experiments (less than 2 μg) would cause the DNA sample to be invisible to the naked eye, some netters say that seeing a pellet of DNA in the bottom of the microcentrifuge tube can be a real psychological boost along the way when several steps of DNA manipulations are to be performed in combination, especially when the cleaning up process involves a precipitation at the end of each step. Most researchers would probably agree that a pellet is a positive sign that things are going well, and can even provoke a sigh of relief that, after rinsing with 70% ethanol, the DNA pellet has not been accidentally washed out of the tube and lost down the sink.

To see a pellet when precipitating very small amounts of DNA, a co-precipitant or carrier can be a real advantage. The type of carrier to be added will depend on what the DNA is to be used for after precipitation, and the following tips should help you in selecting an appropriate one.

Spermine or tRNA. Some people add 0.1 vols of 100 mM spermine to precipitate DNA or use a final concentration of 50 μg ml^{-1} bacterial or yeast transfer RNA as carrier[2,3]. However, spermine does not precipitate DNA below 60 bp long and can be tricky to remove later. Transfer RNA can also be a real problem. In past discussions, one netter reported having a problem when doing RNase protection assays – extra protected bands were routinely seen on gels when bacterial and/or yeast tRNA carrier was used for precipitation of RNA transcripts.

Other netters agree that tRNA is to be avoided, or else extra care should be taken when studies are performed with any type of hybridization reaction, because isolation of DNA or RNA by precipitation with tRNA carrier could cause false positives due to the carry-over of contaminating nucleic acids.

Linear polyacrylamide (LPA) carrier can easily be made by polymerizing a 5% w/v acrylamide solution in 40 mM Tris, 20 mM sodium acetate, 1 mM EDTA (pH7.8), together with 0.01 vols of 10% ammonium persulphate and 0.001 vols TEMED. When the solution becomes viscous (15–30 mins) the polymer is precipitated with 2.5 vols of ethanol and centrifuged for 5 mins when it forms into a clot. The pelleted LPA is dried and 20 vols of sterile water added, then left overnight to swell. Afterwards, the LPA stock is mixed by pipetting. 10 μl of a 1 × LPA (0.25%) or 2 μl of a 5 × (1.25%) LPA stock is added to DNA samples in 100 to 400 μl and 2.5 vols of ethanol added for precipitation[4].

Netters generally use 1 μl of 0.25% LPA and 0.1 vols of 3 M sodium acetate or 0.20–0.25 vols of 10 M ammonium acetate and 2–2.5 vols of absolute ethanol for volumes of DNA solution up to 50 μl. They also advise that the chilling step is unnecessary and that it can generally be disregarded. One person wrote that routinely placing the mixed samples on ice for 15–30 mins, followed by centrifugation for 30 mins at 4°C is more than sufficient in most cases, and if more than 100 ng μl^{-1} of DNA is present, then there is no need for chilling at all and the precipitated DNA can be held at room temperature[5].

David A. Johnston (daj@nhm.ac.uk) wrote that he tested various amounts of plasmid DNA ranging from 600 ng to 1 ng, and determined by comparison on an ethidium bromide-stained agarose gel that there was no apparent loss of DNA when as little as 4 ng were used for precipitation. As this amount of DNA is very close to the minimum that can be detected using ethidium bromide agarose gel electrophoresis, it is likely that even smaller amounts can be recovered. In a study using more sensitive radioactive labelling, it has been reported that 20 bp of DNA in the 20 pg range can

Published by Elsevier Science Ltd

PII: S0968-0004(96)30023-6