Evidence for the metal-cofactor independence of an RNA phosphodiester-cleaving DNA enzyme

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Background: RNA and DNA are polymers that lack the diversity of chemical functionalities that make proteins so suited to biological catalysis. All naturally occurring ribozymes (RNA catalysts) that catalyze the formation, transfer and hydrolysis of phosphodiesters require metal-ion cofactors for their catalytic activity. We wished to investigate whether, and to what extent, DNA molecules could catalyze the cleavage (by either hydrolysis or transesterification) of a ribonucleotide phosphodiester in the absence of divalent or higher-valent metal ions or, indeed, any other cofactors.

Results: We performed *in vitro* selection and amplification experiments on a library of random-sequence DNA that incorporated a single ribonucleotide, a suitable site for cleavage. Following 12 cycles of selection and amplification, a 'first generation' of DNA enzymes (DNAzymes) cleaved their internal ribonucleotide phosphodiesters at rates ~10^7-fold faster than the spontaneous rate of cleavage of the dinucleotide ApA in the absence of divalent cations. Re-selection from a partially randomized DNA pool yielded 'second generation' DNAzymes that self-cleaved at rates of ~0.01 min^{-1} (a 10^8-fold rate enhancement over the cleavage rate of ApA). The properties of these selected catalysts were different in key respects from those of metal-utilizing ribozymes and DNAzymes. The catalyzed cleavage took place in the presence of different chelators and ribonuclease inhibitors. Trace-metal analysis of the reaction buffer (containing very high purity reagents) by inductively coupled plasma-optical emission spectrophotometry indicated that divalent or higher-valent metal ions do not mediate catalysis by the DNAzymes.

Conclusions: Our results indicate that, although ribozymes are sometimes regarded generically to be metalloenzymes, the nucleic acid components of ribozymes may play a substantial role in the overall catalysis. Given that metal cofactors increase the rate of catalysis by ribozymes only ~10^2–10^3-fold above that of the DNAzyme described in this paper, it is conceivable that substrate positioning, transition-state stabilization or general acid/base catalysis by the nucleic acid components of ribozymes and DNAzymes may contribute significantly to their overall catalytic performance.

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Introduction

The discovery in the 1980s of ribozymes, RNA molecules with catalytic activity [1,2], altered perceptions about the ways in which catalysis could take place in biological systems. The discovery that RNA could catalyze biochemical reactions gave rise to the notion of an 'RNA world' [3–5], a stage in evolution in which self-replicating RNA molecules might have constituted a primitive life. Unlike the functionality-rich proteins, however, RNA is a functionality-poor polymer. The monomeric units within RNA — the four nucleotides — are chemically similar to one another, quite unlike the chemically diverse functionalities found in the sidechains of the naturally occurring amino acids. The RNA bases are, moreover, chemically inert; they do not have significant acid/basic properties at physiological pH, nor are they especially good nucleophiles. The 2′ hydroxyl group of the ribose in RNA does provide a potential nucleophile; it appears likely from theoretical considerations, however, that if RNA were to catalyze a broad range of chemical reactions, it would do so by utilizing a variety of cofactors, such as metal ions. Interestingly, all naturally occurring ribozymes have been found to utilize metal-ion cofactors for their catalytic activity [6]. Even so, the catalytic range of these natural ribozymes is restricted to the breakage, formation and transfer of phosphodiester linkages (although it has been proposed recently that RNA catalysis may play a role in the peptidyl transfer step in ribosomal protein synthesis [7]). Because naturally occurring ribozymes are metalloenzymes and RNA is a

functionality-poor polymer, it has been widely speculated that all ribozymes might be regarded as metalloenzymes [6,8,9]. This is an interesting and plausible theory; it would be of interest, however, to investigate the extent to which RNA (and nucleic acids in general) might be able to contribute alone (i.e. in the absence of associated cofactors) to the catalysis of specific reactions. The recent development of *in vitro* selection techniques (SELEX) from random sequence libraries (for reviews see [10–12]) has made the investigation of nucleic acid catalysis possible. Already SELEX has yielded new ribozymes and DNA catalysts (DNAzymes), often for reactions that have not been demonstrated in nature to involve ribozymes (such as conformer isomerization [13], porphyrin metallation [14,15] and halide displacement [16,17]). DNAzymes have also been isolated for a reaction that is catalyzed by natural ribozymes — the cleavage of ribonucleotide phosphodiesters. These phosphodiester-cleaving DNAzymes all utilize metal cofactors, notably, Pb^{2+} [18], Mg^{2+} [19,20], Zn^{2+} [19], Mn^{2+} [19] and Ca^{2+} [21].

A proportion of the novel, SELEX-derived ribozymes and DNAzymes, however, are probably not metalloenzymes [13–17]. Although divalent or other higher-valent cations may be necessary for the correct folding of these RNA and DNA molecules, metal ions are probably not required for their catalytic mechanisms. Of this group, a DNAzyme for porphyrin metallation [14] does not even require divalent cations for its folding. It is theoretically possible that complexly folded nucleic acid catalysts, which also have complex binding and active sites, might be able to catalyze reactions by a number of cofactor-independent strategies, including by increasing effective substrate concentrations, by orientation effects [16,17,22], by substrate destabilization [23], by general acid/base catalysis [24] or by stabilizing the charge and/or shape of the transition state [13–15,25].

Given this range of potential catalytic strategies for nucleic acids, we considered it interesting to investigate whether (or to what extent) nucleic acid catalysts could catalyze the cleavage of an RNA phosphodiester in the absence of any divalent or higher-valent metal cofactor. Initially, we decided to search for DNAzymes that could carry out this catalysis. DNA, lacking the 2′ ribose hydroxyl, is handicapped (compared to RNA) in not having this useful functionality available for acid/base catalysis or to act as a nucleophile. To carry out our investigation, we adapted a well-defined model system. Breaker and Joyce [18,19] successfully used *in vitro* selection to isolate DNA molecules that catalyzed the cleavage of a single internal ribonucleotide phosphodiester in the presence of different divalent metal ion cofactors (Pb^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+}). Recently, this same technology has been extended to derive a new Ca^{2+}- and Mg^{2+}-utilizing DNAzyme [21], and also an efficient DNAzyme for cleaving extended RNA substrates [20].

After 12 cycles of selection and amplification, we obtained a pool of 'first generation' DNAzymes that cleaved their internal RNA phosphodiesters at rates ~10^7-fold faster than the spontaneous rate of cleavage of the dinucleotide ApA under comparable conditions (i.e. in the absence of divalent cations) [26]. These self-cleaving molecules were cloned and sequenced; a single cloned sequence was then resynthesized such that its 40-nucleotide random sequence was randomized further, to allow the generation of DNAzymes with even greater catalytic activity. Re-selection from this mutagenized pool yielded 'second generation' DNA molecules that were able to cleave their internal phosphodiester bond at rates of ~0.01 min^{-1}. The reported half-life for a dinucleotide such as ApA is 4000 years [26]. Our selected DNAzymes therefore enhanced the rate of the order of 10^8-fold over the uncatalyzed rate of cleavage of a single RNA phosphodiester.

Results

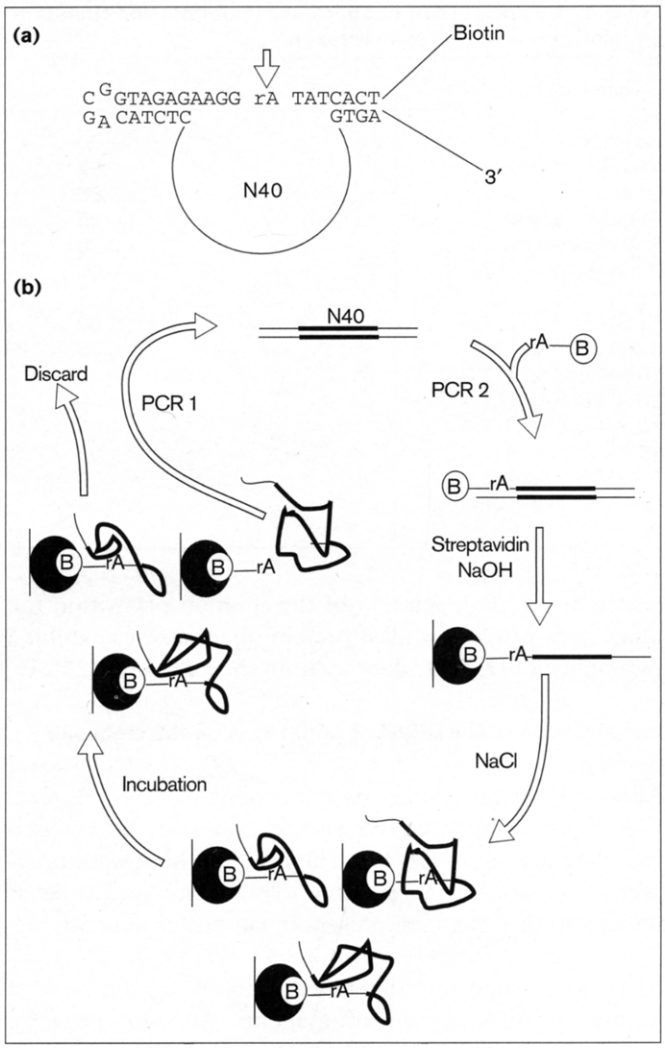
In vitro selection

Our *in vitro* selection strategy, outlined in Figure 1b, was based on a selection scheme designed by Breaker and Joyce [18]. We began selection on a library of ~10^{14} sequences, each of which contained 40 random bases. Figure 1a shows how the random bases were constrained to fold around the target phosphodiester, with the aid of two regions of double-stranded DNA 'clamps'. The selection protocols are described in detail in the Materials and methods section. We continued the rounds of selection until the fraction of released DNA in successive rounds had stabilized at 15% and could not be increased any further.

In the final few rounds of selection, the enriched DNA pools were tested for their ability to self-cleave in reaction buffers supplemented with EDTA. We found that cleavage did proceed in the presence of EDTA comparably to cleavage in the absence of EDTA. This finding gave a preliminary indication that we might have selected for DNA molecules that could catalyze the cleavage of an RNA phosphodiester in the absence of divalent or higher-valent metal ions.

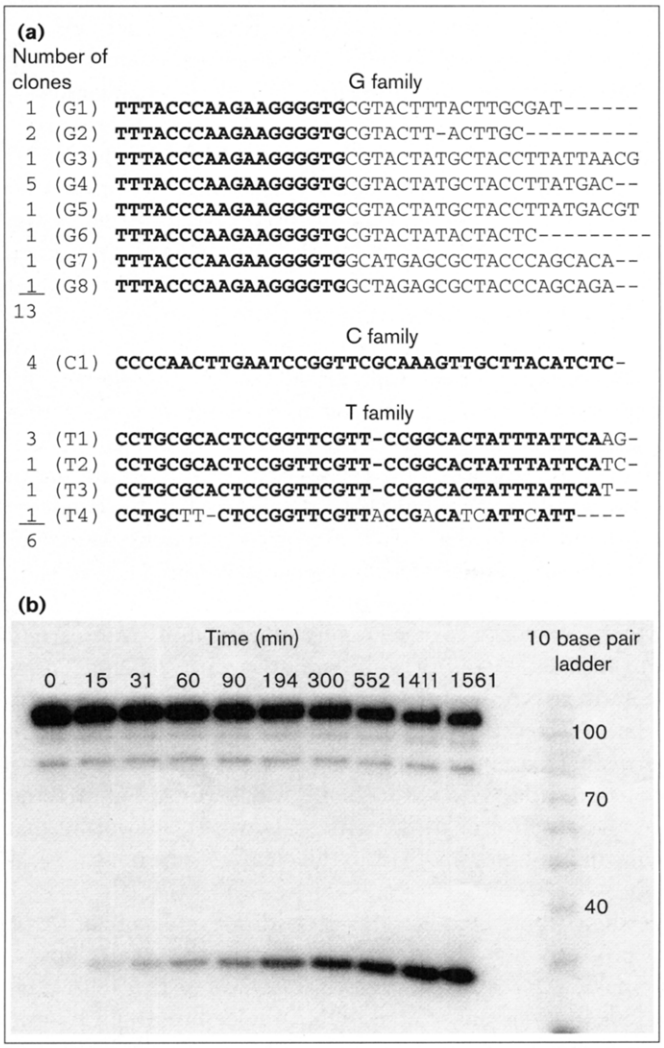
After 12 cycles of selection, the enriched DNA pool was cloned. Figure 2a shows the random 40-nucleotide portions of the sequences of 23 clones, which could be classified into three families on the basis of their sequence (the G, C and T families). Regions of extremely high homology were found among the individual members of all three families (conserved bases shown in bold type in Figure 2a). Preliminary self-cleavage studies carried out with representative clones from each of the three families indicated that the clones from the G family had significantly higher activity than the other two families of clones (preliminary k_{obs}, the observed rate

Figure 1



Design of the random-sequence DNA library and of the selection cycle for self-cleaving DNA molecules. The starting single-stranded random DNA library (107 nucleotides) contains a 5′ biotin attachment (B), an internal ribose residue (rA) and consists of 40 random bases (N40) flanked by two constant regions of 19 and 48 nucleotides. **(a)** The sequences were designed, when folded, to constrain the 40-nucleotide random stretch close to the cleavage site (rA) indicated by the arrow. **(b)** The protocol for the isolation of self-cleaving DNA molecules from the random sequence library. The procedure resembles that used by Breaker and Joyce [18,19], except that here, the incubation of the immobilized DNA molecules was carried out in the absence of added divalent or polyamine cations. The first polymerase chain reaction (PCR 1) amplifies selected sequences from a library of double-stranded DNA with biotin attached to one strand. PCR 2 regenerates the starting form of DNA for each selection step.

Figure 2



Selection and cleavage of clone G3. **(a)** The random-region sequences of DNA molecules were selected for 12 rounds and then cloned. The sequences of the 23 selected clones could be grouped into three families (G, C and T), on the basis of their sequence homologies. The conserved stretches of sequence are shown in bold. Clone G3 was selected for further analysis. **(b)** A time course for the cleavage of clone G3 in reaction buffer (50 mM HEPES, pH 7.0, 0.5 M NaCl, 1 mM EDTA, and 4 μM tRNA carrier). Incubations were carried out at 25°C. The upper band is the uncleaved 107-nucleotide DNA molecule (^{32}P-labelled at its 5′ end). The lower band is the 28-nucleotide cleavage product, which contains the labelled 5′ end of the parent molecule.

for self-cleavage, values in selection buffer at 23°C were of the order of 2×10^{-3} min^{-1}). A number of members of the G family were then tested, and they all showed comparable levels of activity (data not shown). On the basis of these preliminary results, we arbitrarily chose clone G3 for further analysis (Figure 2b shows a gel of the time-dependent self-cleavage of clone G3, under optimized conditions — see below).

Possible roles for buffer composition or protein ribonucleases in the cleavage reaction

Although the selection steps had been carried out in a selection buffer that lacked added divalent or higher-valent cations, it could not be ruled out that trace quantities of such metal ions were present in our selection buffer or that

they were responsible for the observed cleavage activity. We therefore undertook an extensive series of experiments to determine whether the observed cleavage activity was caused by catalysis by buffer components, contaminating protein ribonucleases, or divalent or higher-valent cations.

To investigate whether the buffer used (HEPES) was participating in the phosphodiester cleavage, we measured the k_{obs} of G3 self-cleavage in the presence of increasing concentrations of HEPES (at pH 7.0) and in the presence of fixed concentrations of several other buffers, all at pH 7.0. In the first experiment, the k_{obs} did not increase (within an error of ± 10%) when the concentration of HEPES was varied from 10–200 mM. In the second experiment, the k_{obs} also did not change beyond ± 10% when self-cleavage was carried out in 50 mM concentrations of phosphate, Tris, cacodylate, MOPS, imidazole or lutidine (data not shown). In the light of these results it appeared likely that the buffering components were not contributing to the catalysis of cleavage.

Most of the cleavage reactions investigated were carried out in reaction buffer supplemented with low concentrations of tRNA, bovine serum albumin or glycogen (see the Materials and methods section), to inhibit the adhesion of the single-stranded DNA molecules to the walls of the reaction tube. The cleavage k_{obs} values were not affected by the absence of these carriers, however, indicating that they did not play any part in the cleavage reactions.

A single ribonucleotide present within a molecule of DNA is probably not a good substrate for ribonucleases. Nevertheless, we wished to investigate whether the self-cleavage activity of the selected DNA molecules might be due to contaminating protein ribonucleases. To guard against ribonuclease contamination, cleavage reactions were carried out in buffers made up with diethylpyrocarbonate-treated water [27]. In addition, we examined the effect on G3 cleavage kinetics of the presence of different ribonuclease inhibitors, including human placenta ribonuclease (0.1 U/μl), vanadyl complexes (20 mM), and a combination of the two inhibitors. Table 1 summarizes how, in all cases, the k_{obs} values did not change appreciably in the presence of the inhibitors.

Cleavage reactions were also carried out such that G3, dissolved in 50 mM HEPES, pH 7.0, was first treated with proteinase K, followed by extraction with phenol and with chloroform. NaCl (itself from a phenol-extracted stock solution) was then added to initiate the reaction. Again, this treatment did not substantially affect the k_{obs} value of the cleavage.

Finally, a key piece of evidence favouring the absence of extraneous ribonucleases came from the pH dependence of the cleavage reaction (see below). The cleavage reaction was relatively independent of the solution pH within the range of 5–9, whereas most protein ribonucleases exhibit a pronounced pH dependence within this pH range [28].

Table 1

Effect of the presence of ribonuclease inhibitors and chelators on relative rates of G3 self-cleavage.

Inhibitor/chelator	$k_{relative}$
No inhibitor	1.00
Vanadyl complexes	0.82
RNasin	0.86
Vanadyl + RNasin	0.80
Phenol extraction	0.79
Proteinase K + phenol	0.91
1 mM EDTA, pH 7.0	1.00
5 mM EDTA, pH 7.0	0.95
10 mM EDTA, pH 7.0	0.93
25 mM EDTA, pH 7.0	0.99
10 mM EGTA, pH 7.0	0.92
10 mM DCTA, pH 7.0	1.03
10 mM EDTA, pH 8.0	1.03
Chelex column	1.00

Investigation of the effect of metal ions on the cleavage reaction

Although our preliminary investigations of cleavage reactions had indicated that the reaction proceeded unimpeded in concentrations of EDTA as high as 25 mM, it was important to investigate the possible role of trace quantities of metal ions that might be present in our buffer solutions.

First, we carried out the reaction in the presence of a number of different chelating agents. All solutions were first treated with diphenylthiocarbazone (DPTC; see the Materials and methods section), to remove adventitious and, particularly, thiophilic metal ions [29]. Buffers were then passed through a Chelex column (BioRad), containing metal-binding paired iminodiacetate functionalities. The G3 DNA molecules were then dissolved in treated 50 mM HEPES, pH 7.0, supplemented by various concentrations of EDTA, EGTA, or the strong magnesium and calcium chelator DCTA (*trans*-1,2-diaminocyclohexanetetraacetic acid). In all instances, the DNA solutions were first heated at 90°C for 1 min to denature the dissolved DNA in the presence of the EDTA, EGTA or DCTA and were then cooled to room temperature. Cleavage reactions were subsequently initiated by the addition of NaCl to a final concentration of 0.5 M. Table 1 indicates that the k_{obs} values measured did not change with the DPTC treatment, and that they were independent of the presence of and the concentration of EDTA (from 0–25 mM), EGTA (10 mM) or DCTA (10 mM).

Finally, and most importantly, we studied the self-cleavage reaction using ultra-pure reagents (see the Materials

and methods section). We carried out trace-metal analysis of the ultra-pure buffers using inductively coupled plasma-optical emission spectrophotometry (ICPOES) [30], to confirm the purity of the reagents used. The kinetics of the cleavage reaction carried out under these conditions were essentially indistinguishable from those measured earlier.

Direct measurements of trace metal (i.e. other than Na^+) ion concentrations were carried out using ICPOES [30]. Analysis by ICPOES cannot be performed with high ionic strength solutions such as our reaction buffer. For this reason, the reaction buffer was analyzed containing all its components except the high-purity NaCl (in other words, containing 50 mM Na HEPES, 10 mM EDTA and 15 nM DNA). All cations, other than Na^+, were below the detection limit of the technique (see Table 2). Known concentrations of Mg^{2+}, Ca^{2+} and Pb^{2+} were added back to control solutions to check the accuracy of the analyzing technique. For the ultra-pure NaCl, metal analysis data supplied by the manufacturer (Aldrich) indicated that a 0.5 M solution would contain no more than 0.3 μM Mn^{2+}, and less than 0.25 μM of all other divalent and higher-valent cations. In addition to this, a 25 mM solution of the ultra-pure NaCl analyzed by us using ICPOES revealed that all cations other than Na^+ were below the level of detection (see the Materials and methods section and Table 2).

Given the maximum possible levels of different cations listed above (at less than the limits of detection of ICPOES, see Table 2), we calculated the metal–DNA binding constants that would be necessary for any of the metal ions to populate substantially (in the presence of 10 mM EDTA) a single strong catalytic site within each molecule of the DNAzyme. The details of the formalism used to calculate these predicted DNA–metal association constants are described in the Materials and methods section. All our self-cleavage reactions were carried out such that the metal ions, the DNA and the EDTA in the reaction mixture were at equilibrium prior to the initiation of the reaction with NaCl. Thus, the NaCl-induced folding of the DNAzymes (leading to cleavage) was initiated only after the DNA had first been heat-denatured in the presence of EDTA and then allowed to cool and fold in the presence of EDTA. This was carried out to ensure that metal ions did not remain kinetically bound to hypothetical 'high affinity' binding sites in the DNA, even in the presence of high concentrations of EDTA.

The calculated metal–DNA association constants (Kf_{M-DNA}) necessary for metal ions to be utilized by the DNAzyme under our reaction conditions (in the presence of 10 mM EDTA and assuming a stoichiometry of one metal ion per DNA molecule), are shown in Table 2 (last column). What is striking about these values, ranging from ~10^{10} M^{-1} (for Mg^{2+}) up to ~10^{21} M^{-1} (for Pb^{2+}), is that

Table 2

Metal-ion concentrations in the reaction buffer and predicted metal–DNA binding constants.

Metal ion	Concentration (μM)*	Kf'_{M-EDTA} (M^{-1})	Necessary Kf_{M-DNA} (M^{-1})
Mg^{2+}	< 4.2	3.1×10^5	$> 6.4 \times 10^9$
Ca^{2+}	< 2.5	2.5×10^7	$> 8.5 \times 10^{11}$
Pb^{2+}	< 0.6	5.5×10^{14}	$> 7.8 \times 10^{21}$
Cu^{2+}	< 0.35	3.2×10^{15}	$> 7.8 \times 10^{20}$
Mn^{2+}	< 0.2	3.7×10^{10}	$> 1.6 \times 10^{16}$
Zn^{2+}	< 0.2	1.6×10^{13}	$> 6.8 \times 10^{18}$

*See the trace metal analysis in the Materials and methods section.
Kf_{M-DNA}, formation constant for the DNA–metal ion complex. Kf'_{M-EDTA}, formation constant for the EDTA–metal-ion complex corrected for pH 7.

they are all approximately 4–5 orders of magnitude higher than the Kf' values (at pH 7.0) for the binding of these same metal ions to the excellent chelators, EDTA and DCTA. In other words, for any significant percentage of the DNAzyme molecules, under standard reaction conditions, to have catalytically relevant metal ions bound to them, the DNAzyme–metal affinity (for each of the cations considered) has to be 4–5 orders of magnitude stronger than the binding of the cations to EDTA. The predicted Kf_{M-DNA} values are also 5–7 orders of magnitude larger than the known binding constants for 'strong binding' of metal ions to binding pockets in folded RNA and DNA, such as in tRNA and ribozymes. Thus, for example, the Mg^{2+} defined in a recent crystal structure of the hammerhead ribozyme [31] as binding closest to the catalytic site, and also implicated in another study as being catalytically relevant [32], binds with an association constant of 9 mM (Kf ~10^2 M^{-1}) [32]. A number of other 'strong' Mg^{2+}-binding sites in hammerhead ribozymes have been shown to have a similar affinity [33–35]. A recent SELEX experiment carried out to define folded RNA pockets specific for Zn^{2+}-binding yielded motifs that had 100–400 μM affinities (Kf < 10^4 M^{-1}) [36] for Zn^{2+} (compared to the calculated DNAzyme–Zn^{2+} inter-action of 10^{18} M^{-1}). Two carefully conducted studies report the strongest magnesium–tRNA association constants to be 4.5×10^4 M^{-1} and 7.5×10^4 M^{-1}, respectively [37,38]. Another study measured the strongest Mn^{2+}–tRNA interaction to be 6.2×10^4 M^{-1} [39].

By comparing all of the above data, it is apparent that it is highly improbable that any potential divalent or higher-valent metal-binding sites within the folded G3 DNAzyme might actually be populated by the trace quantities of free (i.e. not complexed to EDTA) metal ions present in our buffers.

We next explored whether deliberate additions of Mg^{2+}, Ca^{2+}, Pb^{2+}, Cu^{2+} and Zn^{2+} to EDTA-free reaction buffers increased the k_{obs} values for G3 self-cleavage. If G3 was

indeed a metalloenzyme (and assuming that any low levels of metals already present had not saturated the catalytically relevant binding sites), the presence of progressively higher concentrations of cations might be mirrored in progressively higher self-cleavage rates. The observed rates (relative to the rate under standard conditions, i.e. in the absence of added cations and in the presence of EDTA) did not change significantly (less than ± 10%) in the presence of 0.01 and 0.1 mM concentrations Zn^{2+}, Pb^{2+} or Cu^{2+}, or with 0.01, 0.1 and 20 mM concentrations of Mg^{2+} or Ca^{2+}. Only the presence of 0.1 mM Cu^{2+} had a measurable and destructive effect on the observed relative rate (60%).

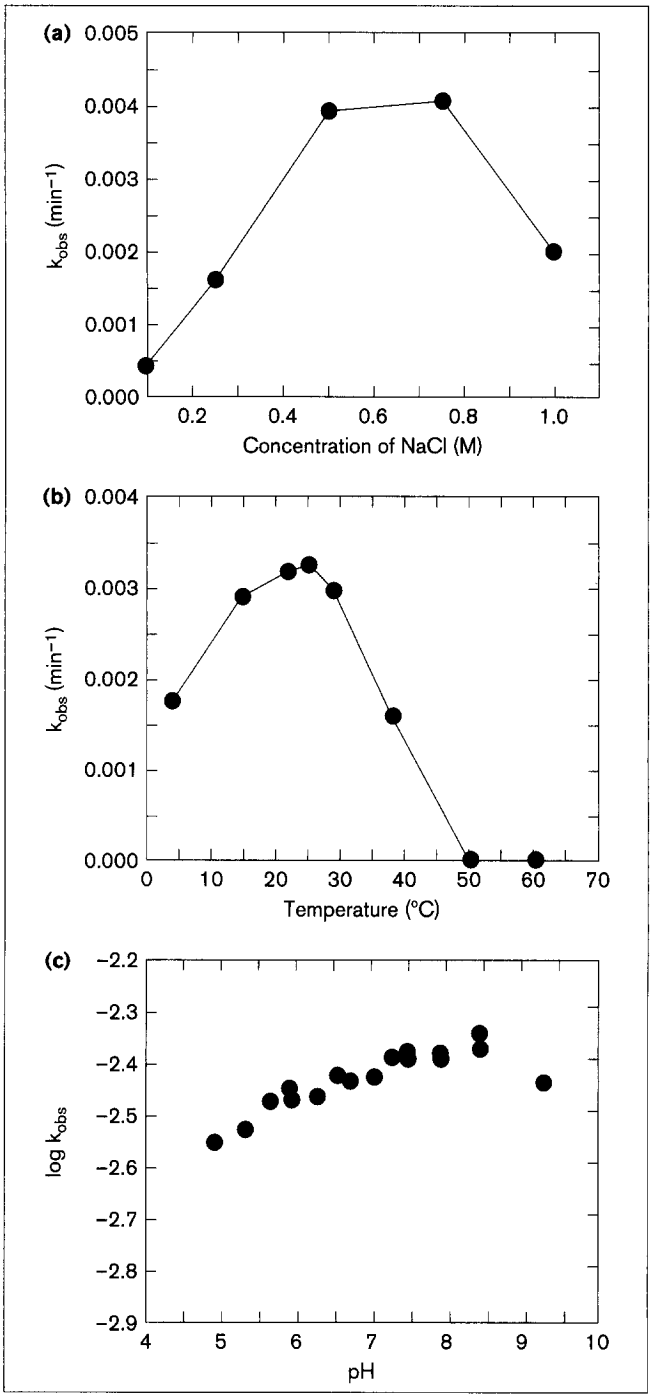
In addition to examining the effect of adding back divalent cations, the cleavage reaction was also studied in the standard reaction buffer, but containing up to 6.5 μM DNA (instead of the usual 15 nM DNA). It was found that, even at this very high DNA concentration, the k_{obs} of the cleavage reaction did not change, suggesting that the self-cleavage activity was not dependent on the limiting presence of a trace metal ion in the standard reaction solution. The rates also did not change substantially (in fact, they showed slight decreases) when either Ca^{2+} or Mg^{2+} was added back to the reaction buffer containing these micromolar DNA samples. Given the very low levels of higher-valent cations in the reaction buffer (<5 μM, see Table 2) and the finding that up to 75% of the total DNA present underwent cleavage (in other words ~4 μM of the DNA in these solutions would have to have a catalytically relevant metal ion bound to it), it becomes very difficult to argue that any higher-valent metal ion, at the levels indicated above (and in the presence of 10 mM EDTA), could possibly be the cofactor for the cleavage.

Characterization of the DNAzyme G3

A preliminary characterization of clone G3 was performed in order to gain insight into how the selected DNAzymes might catalyze phosphodiester cleavage in the apparent absence of divalent and other multivalent cations. We investigated the effects of Na^+ concentration, temperature and pH on the k_{obs} values, as well as the possibility of substituting other cations for Na^+. Figure 3a shows the dependence of k_{obs} on Na^+ concentration. The catalytic rate increased with increasing [Na^+], to a maximum at 0.5 M–0.8 M Na^+; at higher Na^+ concentrations the rate decreased from this plateau. The rather large optimal concentration of Na^+ (in the absence of divalent cations) is not surprising, because nucleic acids fold far less efficiently in the presence of monovalent ions alone. For the remainder of our experiments we chose 0.5 M Na^+ for our reaction buffers.

The dependence of k_{obs} on temperature is shown in Figure 3b. Even in the presence of 0.5 M Na^+, G3 has a low and relatively narrow temperature optimum, centred around 25°C. Above 30°C, the k_{obs} decreased sharply, and essentially no catalysis could be detected at 50°C or above. The relatively low temperature optimum is fully consistent with the relatively inefficient stabilization of the folded, active DNA structure by Na^+.

Figure 3



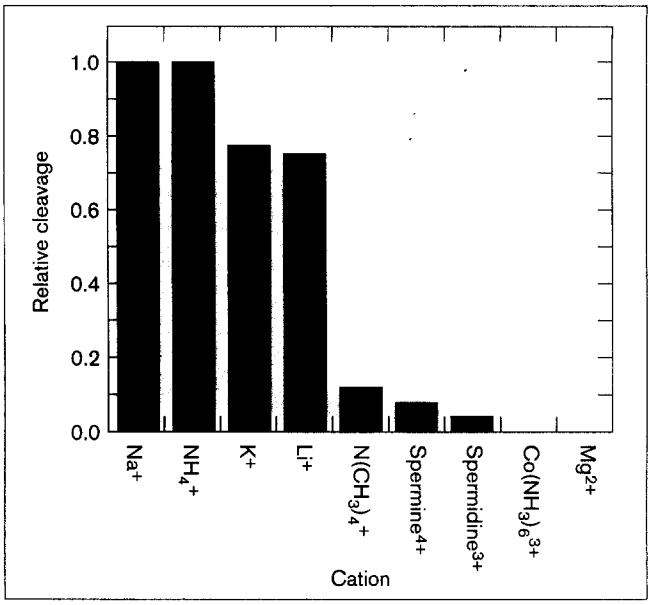
Characterization of clone G3 (see Figure 2a). The reactions were performed in 50 mM HEPES, pH 7.0, 1 mM EDTA, and 0.5 M NaCl, unless otherwise stated. **(a)** Dependence of the observed rate constant for self-cleavage, k_{obs}, on NaCl concentration. **(b)** Dependence of k_{obs} on temperature. **(c)** Dependence of k_{obs} on pH. 50 mM MOPS was used for reactions carried out at pH < 6.0.

Figure 3c shows that the k_{obs} values were essentially independent of pH at pH 5–9. The log of k_{obs} plotted against pH yielded a slope of only 0.05, in marked contrast to the log k_{obs} versus pH plots of both naturally occurring self-cleaving ribozymes (such as the hammerhead ribozyme 40]), those of *in vitro* selected ribozymes (such as the Pb^{2+}-dependent ribozyme [41]) and DNAzymes (including a Mg^{2+}- and Ca^{2+}-dependent DNAzyme [21]), all of which showed slopes of ~1. This linear dependence of k_{obs} on pH was indicative of proton transfer in the rate-determining steps of catalysis by those ribozymes. By contrast, the rate-determining step for catalysis by G3 appears not to involve a proton-transfer step, and its pH profile resembles those of other natural and artificial ribozymes, including the *Neurospora* VS RNA [42], the HDV (hepatitis delta virus) ribozyme [43], and a Mg^{2+}-dependent DNAzyme [19]. The lack of effect of pH values of 5–9 upon the cleavage rate suggests that the rate-limiting step may represent an event, such as a conformational change, prior to the cleavage step.

We investigated whether Na^+ played any specific or necessary role in the activity of G3. Figure 4 indicates the relative rates (relative to rates with 0.5 M NaCl) of G3 self-cleavage in the presence of various cations. In all cases, the reaction buffer consisted of 50 mM Na HEPES, pH 7.0 and 1 mM EDTA. It was supplemented, variously, by 0.5 M of LiCl, KCl, NH_4Cl or Me_4NCl, 20 mM of $MgCl_2$, or 0.1 mM of spermidine, $Co(NH_3)_6^{3+}$ or spermine. In the presence of each of the monovalent cations except Me_4N^+, the k_{obs} values were comparable to that of Na^+; Me_4N^+, however, supported cleavage at only ~10% the rate of Na^+. These results suggest that Na^+ is needed mainly for the folding of the DNA, a role that other monovalent cations can also fulfil. Quarternary ammonium ions, however, are significantly more bulky than the other cations; consequently, they may not be as effective at folding DNA. Interestingly, the cleavage reaction was supported to a small extent (~ 5–10% of that with Na^+) by both spermine and spermidine, but not by $Co(NH_3)_6^{3+}$. Mg^{2+} alone did not support the reaction; conceivably, in the presence of Mg^{2+}, alternatively folded structures from the active one may be formed.

Many of the self-cleaving ribozymes investigated to date (including DNAzymes that catalyze the cleavage of an internal ribonucleotide phosphodiester) [18,19,21,41,42, 44–48] utilize a 2′ hydroxyl as the nucleophile for an in-line attack on the vicinal phosphate. This generates a 3′-end cleavage fragment with a free 5′ hydroxyl and a 5′-end cleavage fragment with either a 3′ phosphate or a 2′–3′ cyclic phosphate. Our analysis of the products of G3 self-cleavage indicated that the 5′ hydroxyl of the 3′-end cleavage product was easily phosphorylated by T4 polynucleotide kinase (under conditions in which only free 5′ hydroxyl groups are phosphorylated; i.e. not under the conditions of phosphate exchange [27]). This was consistent with the findings from experiments with all the other self-cleaving ribozymes and DNAzymes. In addition to this, replacement of the single ribose residue within G3 with a deoxyribose resulted in a complete loss of self-cleavage activity, suggesting that the single 2′ hydroxyl within the G3 molecule was the nucleophile.

Figure 4



Dependence of k_{obs} on various cations. Reactions were carried out in 50 mM Na-HEPES (pH 7.0), 1 mM EDTA, plus the chloride salts of the following cations: 0.5 M Na^+, 0.5 M NH_4^+, 0.5 M K^+, 0.5 M Li^+, 0.5 M Me_4N^+, 0.1 mM spermine^{3+}, 0.1 mM spermidine^{4+}, 0.1 mM $Co(NH_3)_6^{3+}$ and 20 mM Mg^{2+}. The rates shown are reported relative to the cleavage rate in buffer containing 0.5 M NaCl.

Optimization of enzyme activity

The initial, unoptimized, catalyzed rate of G3 self-cleavage (described above) gave a k_{obs} value of $2.8 \pm 0.4 \times 10^{-3}$ min^{-1} (this value varies somewhat depending on the number of cycles of the polymerase chain reaction (PCR) employed to generate the DNA; this phenomenon has also been observed by others [19]). We wished to determine whether this rate could be enhanced. As a first step, we tried to improve the enzyme activity by stabilizing the active folded structure of G3. As reported above, both the high [Na^+] optimum and the low temperature optimum for catalysis suggested that the active enzyme conformation was not very stable. We tried to improve the enzyme's stability by adding various chemicals (salts, detergents and organic solvents) to the pre-existing reaction buffer. Earlier studies with the *Tetrahymena* ribozyme had shown that the addition of ethanol or spermidine could help to stabilize the folded ribozyme structures [49]. In addition, RNA tertiary structures have been proposed to be stabilized by sulfate

anions [50]. We were, however, unable to improve the rate of G3 self-cleavage by adding one of the following individually to the reaction buffer: 20 mM of divalent ions (Mg^{2+}, Ca^{2+} orBa^{2+}), 1 mM $Co(NH_3)_6^{3+}$, 5 mM spermidine, 5 or 25 mM spermine, 20% ethanol, 0.25M Na_2SO_4, 0.5% Tween 20 or 0.5% Nonidet P40 (data not shown).

As we were unable to improve the enzymatic rate of the reaction by the above strategy, we chose to optimize the enzyme's sequence by a re-selection of partially mutated G3. A secondary library of DNA molecules was synthesized on the basis of the G3 sequence (see Figure 2a), but incorporating 15% mutations in the originally random 40 nucleotides. The new molecules had an 85% probability of retaining the original G3 nucleotide at each site within the random region, and a probability of mutation to the other three nucleotides at 5% each. This new library was subjected to an additional six rounds of selection under progressively more stringent conditions (see the Materials and methods section). Following this, the enriched DNA pool was cloned; the sequences of 20 individual DNA clones are shown in Figure 5. As was the case for the DNA sequences of the G family obtained from the initial selection, a strong conservation of certain well-defined regions of sequence was obtained from these mutagenized clones (called the Na family). A consensus sequence derived from these 20 clones is shown at the bottom of Figure 5. The conservation within these stretches is remarkably tight— there are almost no deviations from the consensus, for instance, within the stretch ACCCA AGAAG GGGTG.

A number of the sequences within the Na pools were tested for activity and, of these, Na8 was found to be the most active. The k_{obs} of Na8 was $0.67 \pm 0.07 \times 10^{-2}$ min^{-1}, which represents a modest increase over the k_{obs} measured for the parent sequence, G3. Figure 6 shows the rates of self-cleavage by the clones G3 and Na8. This modest improvement in catalytic rate upon re-selection, as well as the tight conservation of sequences in both the original selection and in the re-selection suggest that the sequences derived are possibly indispensable for this enzyme's activity. The finding that even small sequence deviations from the conserved sequences were not obtained suggests that this folded DNA structure is unique in promoting this particular kind of phosphodiester cleavage.

A structural model for the DNAzyme

From the sequences of the Na family of re-selected clones (Figure 5), we were able to define two conserved regions in the enzyme, one of 18 nucleotides, and another smaller region of five nucleotides. When analyzed by stan-dard RNA-folding programs, the 18 nucleotide cons-erved region folded to form (in the most energetically favourable folded structures) a small hairpin loop with a G–C base paired stem. Figure 7 shows our proposed folded structure for the members of the Na family of

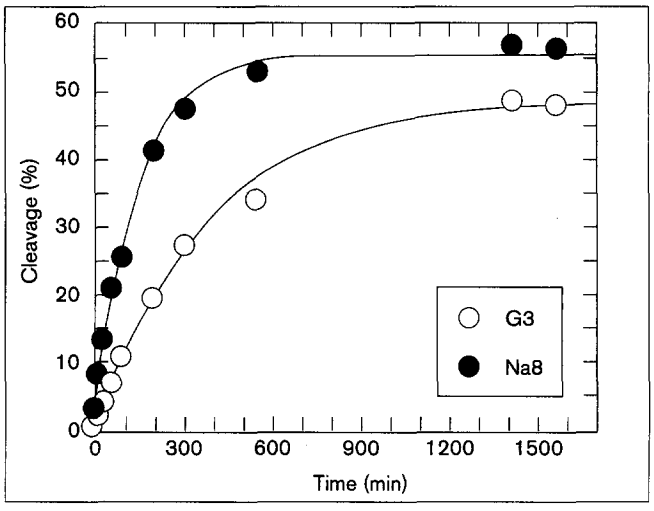
Figure 5

```
G3    TTT--ACCCAAGAAGGGGTGCG--TAC-TAT--GC-T-ACCTTATT-AAC---G-
Na2   TTT--ACCCAAGAAGGGGTGC--cTAC-TtT--GCc--ACCaTATT---------
Na3   TTT--ACCCAAGAAGGGGTGCG--TtCa-AT--GC-T-ACCTTATT-AAC-----
Na4   TTT--ACCCAAGAAGGGGTG-GacT--g-A-gcGCcT-ACCc-Ag-cAga---G-
Na6   TTT--ACCCAAGAAGGGGTGC---TAC-TAT--G-tT-ACCTTATT-A-C---G-
Na9   TTT--ACCCAAGAAGGGGTGCttc-AC-TAT--GC-T-ACgTaATT-AAC-----
Na10  TTT--ACCCAAGAAGGGGTGCG--TAC-TAT-aGCg--ACCgTATT-AAC-----
Na13  TTT--ACCCAAGAAGGGGTGCG-c-AC-TA-ga-C-T-ACCTTAT--AACt----
Na15  TTT--ACCCAAGAAGGGGTGCGg--AC-T-Tg-GCtT--CaTTATT-AAtt--Ga
Na19  TTT--ACCCAAGAAGGGGTGC---TAC-TA--aGCcT-ACtTTATT-AcC-----
Na24  TTT--ACCCAAGAAGGGGTGCaac--C--AT-tGCcTAACCTaAT-cAcC---G-
Na14  TTT--ACCCAAGAAGGGGTGC---TAC--AT--G--T-AC-TTAT--AACttcGa
Na16  ------CCCAAGAAGGGGTGCG--TAaa-cT--GC-T-ACCcTATT-AACct--a
Na1   TTTgtACCCAAGAAGGGGTG--a-TAC-TA-g-GC-T-ACCgTgTT-AAC---G-
Na18  TTTg-ACCCAAGAAGGGGTGC-a-TAC-T-T-aGC-TgACCTTATTcAAC-----
Na22  TTT--ACCCAAGAAGGGGTGC--cTACa-AT--GC-T-ACCcTgTT-cAC---G-
Na23  TTT--ACCCAAtAAGGGGTGC--cTAC-TcT--GC-T-ACgTTATTtAAC---G-
Na17  TT---ACCCAAGAAGGGGTGC--cTA-aT-T--GC-T-AC-TcAca-AAC-----
Na8   TT-g-ACCCAAGAAGGGGTG--acTAC-TtT--GC-T-ACgTTATTccAC-----
      TTT--ACCCAAGAAGGGGTG----------GC-T-AC---AT----------
```

Comparison of the sequences isolated after selection with the randomized sequence of G3 (see Figure 1). G3 was randomized with a degeneracy of 15%. The conserved bases are shown in upper case and the unconserved in lower case. The bottom line shows the consensus sequence, including two highly conserved regions of 15 and 5 bases.

clones. This secondary structure has the attractive feature of locating the targeted ribonucleotide residue close to the putative stem–loop formed by the conserved 18 nucleotide sequence. The other strongly conserved five nucleotide sequence element is found to be exactly complementary to a sequence in the 3′ constant region of the DNAzyme; together, the two elements probably base-pair to form the stem (Stem IV, Figure 7) of a stem–loop structure. Prelimi-

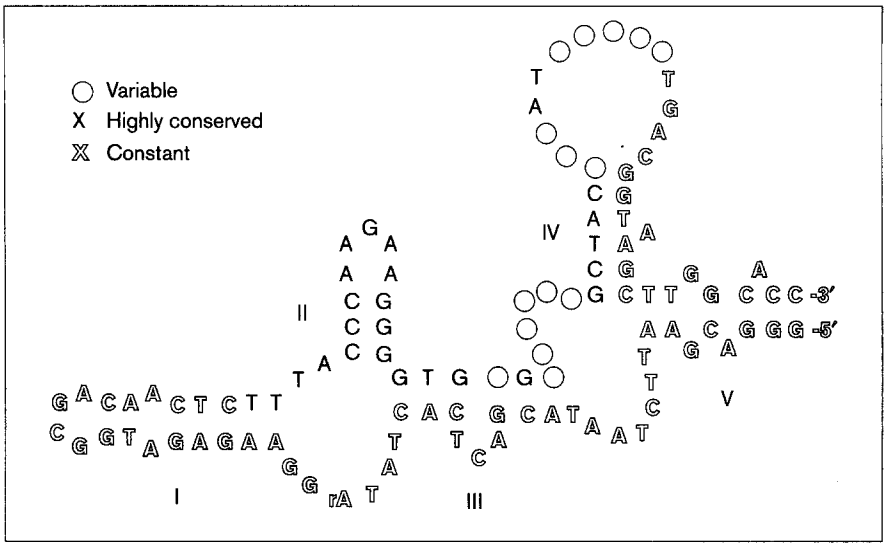
Figure 6



A plot showing the cleavage rates (percentage cleaved as a function of time) of clones G3 and Na8. The data were fit to a first-order rate formalism, using the program GraFit. The data for clone G3 correspond to the gel shown in Figure 2b.

Figure 7

Proposed secondary structure of the Na family DNAzymes. Bold letters indicate the highly conserved bases; open circles denote the variable regions; outlined bases signify the constant sequence 5′ and 3′ regions, originally designed to adopt the secondary structure shown in Figure 1b.



nary studies indicate that Stem IV is essential for the catalytic activity of Na8; deletion mutants lacking it are catalytically inactive. Currently, we are trying to confirm the above structural model via chemical probing experiments and by deletion analysis. A long-range goal, on the basis of detailed secondary and tertiary structural information, is to convert this self-cleaving DNAzyme to a true enzyme that cleaves a distinct substrate molecule *in trans* and is able to turn over substrate.

Discussion

Is the DNAzyme G3 a metalloenzyme?

Several lines of evidence argue against the possibility that G3, Na8 and their relatives utilize divalent or higher-valent metal ions for an essential catalytic role. Experience with known metal-utilizing ribozymes, such as the hairpin and the hammerhead, indicates that the concentrations of metal ions (such as Mg^{2+}) required to achieve maximal activity are minimally of the order of 1–10 mM [44,45]. In all cases, such cations have to be added to the reaction solutions. The measured binding constants of catalytically relevant metal ions that are strongly bound at the ribozyme active sites are only of the order of 10^2–10^4 M^{-1} [32–35]. These modest binding constants are consistent with the finding that the activity of these ribozymes is rapidly and comprehensively quenched by EDTA. Thus, 50 mM EDTA was used to quench reactions containing 12 mM $MgCl_2$ and 25 mM $CaCl_2$, respectively [44,45]. By contrast, we have shown here that the self-cleavage reactions of the G3 and Na8 DNAzymes, in reaction buffers containing < 5 μM of any divalent cation, work comparably well in the absence or presence of EDTA (25 mM) or equivalent chelator. So, if G3 is a metalloenzyme, it is of a radically different nature from the hammerhead ribozyme or from a host of *in vitro* selected ribozymes and DNAzymes. If G3 does utilize a divalent or higher-valent metal ion, it must have a metal-binding site with a metal affinity that is 5–7 orders of magnitude greater than any measured thus far.

Our observations that adding back cations such as Mg^{2+}, Ca^{2+}, Pb^{2+}, Zn^{2+} and Cu^{2+} did not significantly influence the rate of G3 self-cleavage are consistent with the above notion. The predicted G3–metal binding constants are 4–5 orders of magnitude higher than the binding constants for the same metals to even such excellent chelators as EDTA and DCTA [51]. EDTA binds to divalent and trivalent cations essentially by establishing six direct inner sphere coordinations, by its own functionalities, to the metal ion. A determination of the crystal structure of an EDTA–Mn(II) complex indicates four Mn–O distances and two Mn–N distances that are all < 2.3 Å [52]. A body of evidence indicates, however, that the strong binding of metal ions to nucleic acid pockets is fundamentally different from the binding of metal ions to EDTA, with only 0–2 direct coordinations (defined by M–N and M–O distances being < 2.3 Å) between phosphate anionic oxygens and the metal ion [31,47,53]. The remaining contacts between RNA and metal ions are indirect or outer sphere — chiefly hydrogen bond interactions between metal-bound water molecules and various RNA functionalities. For example, a catalytically important Mg^{2+} in the hammerhead ribozyme binds with an affinity of ~10^2 M^{-1}; a recent crystal structure [31] indicates that it is bound to the catalytic site as $Mg(H_2O)_6^{2+}$, with no direct coordination to any part of the RNA. A tRNA-cleaving Pb^{2+} bound to yeast phe-tRNA has one, or at most two, strong (~2.5 Å) oxygen–metal distances [53]. As discussed in the Results section, the most accurately measured association constants for the strongest Mg^{2+}–tRNA

and Mn^{2+}–tRNA interactions are of the order of ~10^5 M^{-1} [37–39]. (It is interesting to note that these binding constants were measured in far lower ionic strength solutions than our 0.5 M NaCl-containing buffers. In 0.5 M salt buffers, the binding of divalents would be expected to be significantly weaker [37].) In summary, there is absolutely no precedent in the literature for the existence of the powerful metal-binding sites in RNA or DNA that would be necessary for G3 to be a metal-dependent DNAzyme.

For our calculations, we have assumed that metal ions, DNA and EDTA exist at equilibrium in our reaction buffers prior to the initiation of self-cleavage. To ensure that this was so, we heat-denatured all our DNA molecules in the presence of 10 mM EDTA, and allowed them to fold in that EDTA. Workers with certain metalloenzymes have occasionally reported high kinetic stabilities (i.e. very slow dissocation rates) of protein-bound, catalytically relevant metal ions [29]. Metal-ion association rates with RNA and DNA are, however, very fast, close to the rate of diffusion [54], implying that dissociation rates from G3 would have to be 10^5–10^7-fold slower than all other metal dissociation rates from nucleic acids reported to date. The rapid quenching with EDTA of the activities of all known metalloribozymes testifies to the rapidly reversible nature of Mg^{2+}-binding to RNA and DNA. The argument against kinetic effects is also supported by the fact that G3 and its relatives are synthetic oligonucleotides (i.e. are not purified from an organism with a bound metal ion already in place), and that they are rigorously heat-denatured (to destroy any metal-binding pockets) in the presence of EDTA, and then allowed to cool and renature in the presence of EDTA.

The dependence of the reaction rates of extant ribozymes (all metalloenzymes) on cofactor-ion concentration is very different from the situation we report with G3. Ribozymes such as the hammerhead and the hairpin generally require Mg^{2+} for two purposes: to fold the ribozymes into their active conformations and for the actual catalytic mechanism of self-cleavage. Spermidine or spermine can replace Mg^{2+}, at least for the above two classes of ribozyme, in its folding role. This permits the study of what is putatively the ribozymes' catalytic rather than structural requirement for Mg^{2+}. A spermine-folded hammerhead ribozyme reaches maximal activity at ~20 mM Mg^{2+} (with half-maximal activity at 3–4 mM) [44]; the spermidine-folded hairpin requires 10 mM Mg^{2+} (with half-maximal activity at ~3 mM) [45]. Even two SELEX-derived DNA metalloenzymes (that catalyze the cleavage of a single, internal, phosphodiester), derived in a very similar manner to G3, have the following metal requirements. A Mg^{2+}-utilizing DNAzyme, folded with 1 M NaCl, functions maximally at 50–100 mM Mg^{2+} (half-maximal rate at 10 mM) [19]; a recently reported Mg^{2+}- and Ca^{2+}- dependent DNAzyme, folded with ~0.3 M total of alkali metal salts, saturates at ~10 mM Ca^{2+} (half-maximal rate at ~2 mM) [21]. In contrast, G3 functions at similar rates through large concentration ranges of many different divalent cations (see above).

Since the first descriptions of catalytic RNA, a number of small and modestly self-cleaving RNA motifs have been described, some of which utilize metal ions for their cleavage [41,55,56], whereas others have been reported that appear not to require divalent metal ions for their cleavage. Of the latter, Watson *et al.* [57] reported a self-cleaving RNA hairpin, PSSp1, from T4-infected *Escherichia coli* cells, which underwent cleavage in the absence of both ribonucleases and divalent metal ions. This cleavage, however, was dependent on the presence of both NH_4^+ and non-ionic detergents (Brij 58, Nonidet P40 or Triton X-100). In another instance, Kierzek [58] reported that a number of RNA molecules cleaved preferentially at UA sites in the presence of a variety of (non-ribonuclease) proteins and also in the presence of polyvinylpyrrolidone. In contrast to these above cases, however, G3 self-cleavage was not found to be sensitive to the presence of non-ionic detergents (Nonidet P40, Triton X-100) or bovine serum albumin.

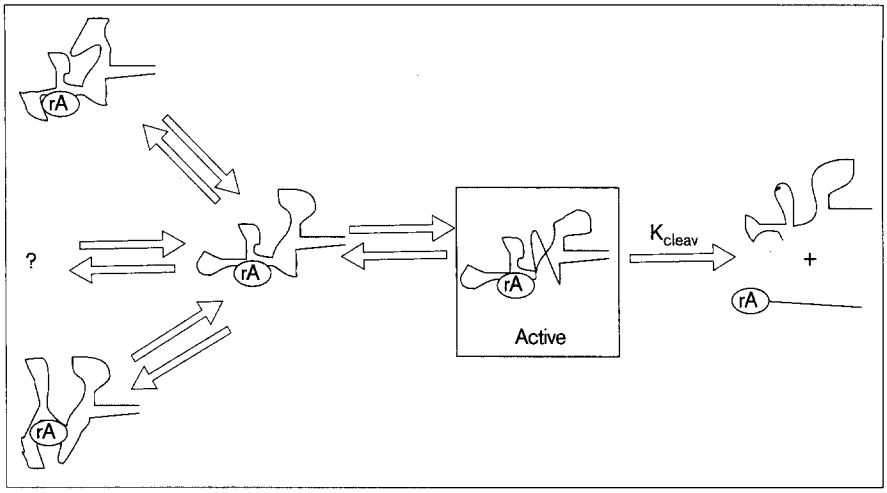
A comparison of the conserved sequences in the G and Na families with the sequences of other *in vitro* selected DNAzymes revealed one interesting homology. Faulhammer and Famulok [21] have recently reported a series of Ca^{2+}- and Mg^{2+}-dependent DNAzymes for phosphodiester cleavage, obtained using a very similar technology to that used here. A total of 44 clones (belonging to eight distinct sequence families) was reported. A conserved 22–24 nucleotide sequence motif which was possibly a metal-binding domain was shared by four of the eight families (representing 35 out of the 44 sequenced clones). Interestingly, one of the minor sequence families (five clones) from this selection, which did not contain the above motif, did contain the following sequence: 5'...TACCC AAGAA GGGGT G..., which is identical to a portion of the major conserved motif in our G and Na families of DNAzymes (see Figures 2 and 5). Faulhammer and Famulok did not report the properties of this family of clones. We propose, however, that this family probably represents a class of metal-independent DNAzymes. That these DNA molecules should have been co-selected with the other, conventional metal-dependent DNAzymes is not entirely surprising; the authors carried out their *in vitro* selection in ~0.3 M alkali halides (probably sufficient for DNA folding), and in the presence of a very small concentration of Mg^{2+} (0.5 mM).

Implications for metal-independent catalysis by nucleic acid enzymes

The monovalent-cation-dependent DNAzyme, G3, provides a rate enhancement of ~10^8 over the cleavage of an RNA dinucleotide in the absence of divalent metal ions [26]. It is conceivable, however, that the rate enhancement

Figure 8

A kinetic model for the observed catalysis. The DNAzyme molecules containing a ribonucleotide rA, as folded in 0.5 NaCl, probably populate a number of interconverting folded forms, only one (or a small number) of which has the active conformation for self-cleavage. The rate-limiting step for the self-cleavage is therefore seen to be the rate of conformational change from inactive folded forms to the active form.



for the chemical step is actually higher than this, given that the observed rate-limiting step (above) for G3 appeared not to reflect the chemical step. We postulate that the rate-limiting step in the reaction represents a conformational switch, illustrated in Figure 8. In this case, the lowest ground state for the enzyme is not the catalytically active conformation; the DNAzyme must undergo a slow conformational change before the reaction can proceed.

A rate enhancement of 10^8 is a surprisingly large one, considering the lack of any conventional acid/base catalysts in the experimental system (including the reaction buffer, which supplied no divalent or higher-valent metal ions and no polyamines to the reaction). We propose that the DNAzyme, in its active conformation, facilitates the reaction by utilizing one or more of the following strategies: supplying one or more of its bases, with altered pK_a values, for general acid/base catalysis; precise positioning of reactive groups, such as the 2′ hydroxyl nucleophile for a favourable in-line attack; or stabilizing the pentacoordinate phosphate through hydrogen bonds.

We compared the observed cleavage rates of G3 and Na8 with those of metal-aided ribozymes— both natural and *in vitro* selected. Under optimal (but not necessarily physiological) conditions, hammerhead ribozymes (as well as other natural ribozymes) can have k_{obs} values > 1.0 min^{-1} (reviewed in [59]). Remarkably, a series of magnesium-aided self-cleaving ribozymes selected *in vitro* by Williams *et al.* [55] had lower k_{obs} values (0.003 min^{-1}) than Na8. The metal-dependent, phosphodiester-cleaving DNAzymes that have been previously reported had k_{obs} of 0.01 min^{-1} [19] 0.03 min^{-1} [21] 0.2 min^{-1} [18] and 4 min^{-1} [20]. G3 and Na8 were therefore 1–3 orders of magnitude slower than the above metal-aided DNAzymes and, typically, 2–3 orders of magnitude slower than the hammerhead ribozyme. Perhaps the most significant enzymatic parameter for G3 and Na8, however, is not their absolute catalytic rates but rather the rate acceleration they provide over the uncatalyzed rate of cleavage of a dinucleotide (under comparable ionic conditions). This acceleration is of the order of 10^8; in this respect, G3 and Na8 are quite superior catalysts.

To summarize, this study suggests that in ribozyme and DNAzyme systems, the nucleic acids may play a considerable role in the catalysis, rather than just acting as passive scaffolds for the binding of metal-ion cofactors. The nucleic acids may provide binding energy to facilitate the in-line attack of the 2′ hydroxyl on the vicinal phosphate. Alternatively, the nucleic acids may stabilize the transition state through hydrogen bonding or even supply functionalities that contribute to general acid/base catalysis. This study reports the remarkable finding that the nucleic acids in themselves are able to provide rate enhancements of ~10^8 over an uncatalyzed cleavage reaction in the presence of cations that appear to assist only the folding of the DNA. The finding that nucleic acids, with no associated cofactors, can contribute significantly to the cleavage of phosphodiesters should change our perceived view of the enzymatic requirements for RNA cleavage. Finally, we believe we have provided compelling evidence that the phosphodiester-cleaving DNAzymes that we have isolated function without divalent or higher-valent metal ions. If these DNAzymes were actually to be metalloenzymes, they would constitute a radically different class from all natural and artificial ribozymes studied to date, by virtue of having a catalytically relevant metal ion-binding site to which metal ions bind with an unprecedented strong affinity.

Significance

Here, we report the properties of a phosphodiester-cleaving DNAzyme (DNA catalyst), G3. Our findings indicate that G3 function is not dependent on divalent or

higher-valent metal-ion cofactors. The activity of G3 is unaffected by the presence of high concentrations of various chelators. The possibility that catalysis is influenced by ribonuclease contamination and buffer components in these studies has been ruled out, and trace-metal analysis by inductively coupled plasma-optical emission spectrophotometry indicates that a plausible case for metal-ion-mediated catalysis by G3 cannot be made. Although catalysis by G3 is two to three orders of magnitude slower than that by natural ribozymes, G3 nevertheless appears to be an excellent catalyst, providing a rate acceleration of ~10^8 over the uncatalyzed rate of hydrolysis of a dinucleotide in the absence of divalent ions. Our results suggest that in ribozymes and DNAzymes the nucleic acid components, exclusive of any cofactor, may have more important roles than just acting as passive scaffolds for the binding and positioning of metal-ion cofactors.

Materials and methods

Materials

Random DNA oligonucleotides were purchased from DNAgency (Malvern, PA). Primers were from University Core DNA Services, (Calgary, AB). *Taq* polymerase was purchased from Gibco. Proteinase K, Human placenta ribonuclease inhibitor, Vanadyl complex were from Sigma. T4 polynucleotide kinase and bovine serum albumin were from New England Biolabs. Cloning kits were from 5 prime-3 prime. γ-^{32}P-ATP and α-^{32}P-ATP were from Amersham. Streptavidin columns were from Genosys. Chelex 100 deionizing resin was from BioRad. T7 DNA Sequencing kits were from Pharmacia.

Ultra-high purity reagents were purchased from Aldrich and from Sigma. These included sodium chloride (Aldrich– 99.999% pure; Mn^{2+} at 0.7 ppm; Ag^{+} at 0.6 ppm; all others below 0.5 ppm); sodium hydroxide, HEPES (HEPES free acid and sodium salt) and EDTA (Sigma; all containing Ca at < 10 ppm; Cu, Mg, Mn, and Pb at < 5 ppm); and water (Sigma; no metal ions detected by ICP analysis).

Preparation of a random DNA library

A 40 random base synthetic oligomer was synthesized on automated DNA synthesizer (5′-GTGCC AAGCT TACCG TCAC-N_{40}-GAGAT GTCGC CATCT CTTCC TATAG TGAGT CGTAT TAG-3′) and amplified in a 10 ml PCR reaction using primers JDP1: 5′-GTGCC AAGCT TACCG-3′ and JDP2: 5′-CTGCA GAATT CTAAT ACGAC TCACT AT AGG AAGAG ATGGC GAC-3′. The resulting PCR products were gel purified on 6% denaturing polyacrylamide gels and stored as stock library. The gel-purified DNA (14 µg) was amplified in a 2.5 ml PCR reaction, which contained the 3′ribose base primer, RAP4bio: 5′-biotin-GGGAC GAATT CTAAT ACGAC TCACT AT(rA)-3′; and the primer, JDP1 (see above). The PCR products were gel purified on a 6% denaturing polyacrylamide gel. PCR conditions for the above reactions were as follows: 0.05 U µl^{-1} *Taq* polymerase, 0.2 mM dNTPs, 1 µM DNA primers, 10 mM Tris (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$. The PCR cycles consisted of the following steps: 45 s at 94°C; 1 min at 50°C; and 2 min at 72°C. Following the PCR amplification, the products was phenol-chloroform extracted and ethanol precipitated.

In vitro *selection*

The *in vitro* selection protocol is shown schematically in Figure 1a. The starting pool of random DNA was dissolved in 0.5 ml of binding buffer (1 M NaCl, 50 mM HEPES, pH 7.0) and divided among four streptavidin columns and incubated for 30 min. The unbound DNA was washed off the column with 1.5 ml of binding buffer. Each column was then rinsed with 5 × 100 µl of 0.2 N NaOH (prepared fresh) to remove the non-biotinylated strand. The column was neutralized with 1 ml of 50 mM HEPES, pH 7.0. The cleavage reaction was initiated by incubating the column in 50 µl of reaction buffer (1 M NaCl, 50 mM HEPES, pH 7.0) at room temperature (23°C). The incubation was carried out for 1 h. Every 20 min, the reaction buffer in the column was expelled and replaced by 25 µl of fresh reaction buffer. The eluted DNA was ethanol precipitated in the presence of 40 pmol of JDP1 and JDP2. Following the precipitation, the DNA was suspended in 100 µl of 10 mM Tris (pH 8.3), 0.1 mM EDTA. A 50 µl trial PCR was initiated using 25 µl of the selected DNA to determine the number of cycles required. Following the trial PCR, 50 µl of the selected DNA was used to initiate a 100 µl PCR (PCR1) reaction, using the number of cycles determined in the trial PCR. 10 µl of PCR1 was used to initiate a 100 µl PCR (PCR2) reaction containing 100 pmols of primers RAP4bio and JDP1 in the presence of 20 mCi (α-^{32}P-dATP). PCR2 was amplified for eight cycles. The PCR reaction was phenol-chloroform extracted and ethanol precipitated. The precipitated DNA was suspended in 50 µl of binding buffer and the selection repeated. If the product DNA from the trial PCR1 did not appear as a well-defined band, then the DNA was gel purified after PCR1 before proceeding further.

In vitro *selection of a partially mutagenized library*

A degenerate library based on clone G3: 5′-CTGCA GAATT CTAAT ACGAC TCACT ATAGG AGGAG ATGGC GAC[TT TACCC AAGAG GGGTC GCTAC TATGC TACCT TATTA ACG]TT GACGG TAACG TTGGC AC-3′ was synthesized with each of the bases within the brackets occurring as the base indicated with an 85% probability, and as each of the other nucleotides with a 5% probability. The selection was carried out in the same manner as the first selection except that the reaction buffer contained only 0.5 M NaCl, and incubation times were decreased as the selections proceeded. Thus, rounds one and two were incubated for 1 h; round three for 30 min; round four for 10 min; and, rounds five and six for 1 min each.

Cloning and sequencing

The DNAs from the selection were amplified by PCR with primers JDP1 and JDP2. The PCR products were blunt-end cloned using the Prime PCR Cloner Cloning System (5′–3′). The individual clones were sequenced using the Pharmacia T7 Sequencing Kit.

Kinetic analysis

Clones to be tested for activity were prepared as follows: sequences were generated by PCR amplification from the plasmid using primers JDP1 and JDP2. After PCR, the DNA was gel purified, and a second PCR was performed using primer RAP4 to introduce the single ribonucleotide and primer JDP1bio to introduce the 5′ biotin. The PCR products were phenol-chloroform extracted, ethanol precipitated, and then dissolved in binding buffer (50 mM HEPES, pH 7.0) and applied to a streptavidin column. The column was washed with buffer to remove unbound DNA, and the catalytic strand of DNA eluted from the column using 3 × 100 µl of 0.2 M NaOH. Following neutralization with 60 µl of 3 M NaOAc, the DNA was precipitated, and kinased using T4 polynucleotide kinase and γ-^{32}P-ATP. After a step of gel purification, the kinased DNA molecules were suspended in 50 mM HEPES, pH 7.0, and stored in aliquots at −80°C.

Standard self-cleavage assays were carried out in the following reaction buffer: 50 mM HEPES, pH 7.0, 1 mM EDTA, 0.5 M NaCl, and 0.1 µg ml^{-1} tRNA. The reactions were carried out at 25°C. First, the DNA, dissolved in the above buffer (minus the NaCl), was heated to 90°C for 1 min, and then slowly cooled to room temperature. The cleavage reactions were initiated by adding NaCl from a stock to 0.5 M, and quenched by tenfold dilution in denaturing running buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 95% formamide). Reaction products were separated in a 10% denaturing polyacrylamide gel, and gel bands quantified using a BioRad Phosphorimager. The tRNA was added to the reactions to prevent the DNA from adhering to the tube. However, similar experiments conducted in the absence of tRNA, as well as in the presence instead of tRNA of bovine serum albumin (50 mg ml^{-1}),

or glycogen (0.1 mg ml^{-1}), gave similar k_{obs} values in all cases, thus confirming that the tRNA did not play any part in the catalysis.

Removal of metal-ion contaminants

All reaction solutions were treated with DPTC as described in [44,51]. All solutions were individually extracted with equal volumes of 15 μM DPTC in water-saturated chloroform. The samples were vortexed with the DPTC solution and incubated for 10 min at room temperature. Following the incubation, the samples were centrifuged to recover the aqueous phase. Following DPTC treatment, the cleavage reactions were performed in conditions identical to the standard conditions (see section on kinetic analysis), except one of the following chelators was added: EDTA at 5, 10 and 25 mM; EGTA at 10 mM; or DCTA, at 10 mM. For the reactions where divalent metal ions were added back to the reaction, one of the following metal ions was used at a time: $CuCl_2$, $ZnCl_2$, $PbCl_2$ and $MgCl_2$. The reaction conditions were identical to the standard conditions except that 0.01 mM, or 0.1 mM, of $CuCl_2$, $ZnCl_2$, $PbCl_2$ (or 0.1 mM and 1.0 mM of $CaCl_2$ or $MgCl_2$) were added, in the absence of EDTA.

Solutions pre-treated with Chelex 100 resin were incubated with the resin for 1 h at room temperature (5 g Chelex resin for every 50 ml of solution). Following the incubation, the solutions were passed through a column containing more Chelex resin (again, 5 g for every 50 ml of solution).

Trace-metal analysis

Trace-metal ion analysis in the buffer solutions, using ICPOES, was carried out at Analytical Service Laboratories, Vancouver, B.C.). The detection limits by ICPOES (also shown in Table 2, column 2) are as follows: Ca, 2.5 μM; Mg, 4.2 μM; Pb, 0.6 μM; Mn, 0.2 μM; Cu, 0.35 μM; and Zn, 0.2 μM. Analysis with ICPOES cannot be performed with high ionic strength solutions such as our reaction buffer. For this reason, the reaction buffer was analyzed in the following way: first, it was analyzed containing all its components except the high-purity sodium chloride (in other words, containing 50 mM Na HEPES, 10 mM EDTA, and 15 nM DNA). For estimating possible metal ion contaminants in the ultra-high purity sodium chloride that we used, we utilized the figures for the upper limits of impurities measured and specified by Aldrich in its quality control certificate issued with the ultra-high purity sodium chloride. In addition, we confirmed that a 25 mM solution of this sodium chloride had contaminating metal ions below the detection limits of ICPOES (above).

Ribonuclease contamination

The reactions were performed under standard conditions, except for the following. The water for the reactions was treated with diethylpyrocarbonate (DEPC) prior to use [28]. When human placenta ribonuclease inhibitor was used it was added at 0.1 U μl^{-1}. Vanadyl complexes, when used, were added to 20 mM. The proteinase K treatment and phenol extractions were performed in a standard way, and are described in [28].

Cation experiments

Reactions testing for the effect of cations other than NaCl were performed as under standard conditions, except that the cations were added in place of NaCl. The anion used was always Cl^-, except for the experiment testing sulfate anions.

Calculations

The 1:1 stoichiometric binding of metal ions to EDTA is defined by formation constants, K_f. However, a more appropriate measure of the binding affinity of different metal ions to EDTA is the pH-dependent, 'effective formation constant', K_f', related to K_f by the formula: $K_f' = \alpha K_f$. The constant α represents the molar fraction of the tetra-anionic species present at a given pH. The α factor is 5.0×10^{-4} for EDTA at pH 7.0 [51,60]; K_f' values for Mg^{2+} and Ca^{2+} ions at pH 7.0 are therefore 3.0×10^5 and 2.5×10^7, respectively [51,60]. Although DCTA is intrinsically a stronger chelator of magnesium and calcium (with higher K_f values) than EDTA, its larger α-factor results in both EDTA and DCTA having, at pH 7.0, approximately the same K_f' values for magnesium (3.7×10^5), and for calcium (5.6×10^7) [61]. EGTA is a poorer binder of either ion than EDTA and DCTA.

The binding of metal ions to EDTA and to DNA in competition can be described in terms of the following three simultaneous equilibria:

$$M + EDTA \overset{K_{EDTA}}{\rightleftharpoons} M\text{–}EDTA \quad (1)$$

$$M + DNA \overset{K_{DNA}}{\rightleftharpoons} M\text{–}DNA \quad (2)$$

$$Na + EDTA \overset{K_{Na}}{\rightleftharpoons} Na\text{–}EDTA \quad (3)$$

Although the interaction of EDTA with sodium ions is weak, we nevertheless make allowance for a proportion of EDTA that may be complexed to Na^+ (owing to the high concentration (0.5 M) of Na^+ used in the reaction buffers). From the above formalisms, we can write the following expressions:

$$K_{Na} = \frac{[Na-EDTA]}{[Na_{free}] \cdot [EDTA_{free}]} \quad (4)$$

Therefore:

$$[EDTA_{free}] = \frac{[Na-EDTA]}{K_{Na} \cdot [Na_{free}]} \quad (5)$$

Also:

$$K_{EDTA} = \frac{[M-EDTA]}{[M_{free}] \cdot [EDTA_{free}]} \quad (6)$$

If we substitute for $[EDTA_{free}]$ from Equation 5, we have:

$$K_{EDTA} = \frac{K_{Na} \cdot [M-EDTA] \cdot [Na_{free}]}{[M_{free}] \cdot [Na-EDTA]} \quad (7)$$

Now, K_{DNA}, the unknown to solve for, is given by:

$$K_{DNA} = \frac{[M-DNA]}{[M_{free}] \cdot [DNA_{free}]} \quad (8)$$

Substituting for $[M_{free}]$ from Equation 7, we have:

$$K_{DNA} = \frac{K_{EDTA}[M-DNA] \cdot [Na-EDTA]}{K_{Na} \cdot [DNA_{free}] \cdot [M-EDTA] \cdot [Na_{free}]} \quad (9)$$

Given that $[EDTA_{total}] = 10$ mM and $[Na_{total}] = 500$ mM, we can say that $[Na_{free}] = [Na_{total}]$. Also, given that there is an enormous excess of EDTA compared to DNA (15 nM) or to metal ions ($[M] < 5$ μM), $[EDTA_{total}] >> [EDTA\text{–}M]$. Therefore $[EDTA_{total}]$ approximately equals $[EDTA_{free}]$ + [EDTA–Na]. Now, Kf'_{Na} equals 2.29×10^{-2} M^{-1} [60]. Utilizing equation (4), therefore, we have [Na–EDTA] = 0.11 mM when $[EDTA_{total}] = 10$ mM.

As mentioned above, there is an enormous excess of EDTA over the competing chelator, DNA (15 nM), and over metal ions ([M] ~5 μM),

[M–EDTA] >> [M–DNA], and therefore, [M–EDTA] ~ [M_{total}]. We then have, re-arranging Equation 9:

$$K_{DNA} = \frac{[M-DNA]\cdot(0.11\times10^{-3})\cdot K_{EDTA}}{[DNA_{free}]\cdot[M_{total}]\cdot(0.5)(2.29\times10^{-2})} \quad (10)$$

For a conservative estimate of K_{DNA}, we can assume that approximately 90% of the DNA molecules, at equilibrium and in the presence of 10mM EDTA, are able to bind a metal ion (the extent of maximum cleavage of individual DNA samples was typically 75% – see above). In that case, [M-DNA] / [DNA_{free}] would be 9. Therefore, for any given cation, we would have:

$$K_{DNA}(\text{at 90\% saturation}) = \frac{(0.086)K_{EDTA}}{[M_{total}]} \quad (11)$$

Note added in proof

Faulhammer and Famulok (*J. Mol. Biol.* (1997) **269**, 188-203) have recently proposed that a family of DNAzymes that they derived [21], which have substantial sequence homology with G3 and Na8, function without added metal ions.

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