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In Vitro Selection of High Temperature Zn^{2+}-Dependent DNAzymes

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Abstract. *In vitro* selection of Zn^{2+}-dependent RNA-cleaving DNAzymes with activity at 90°C has yielded a diverse spool of selected sequences. The RNA cleavage efficiency was found in all cases to be specific for Zn^{2+} over Pb^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Hg^{2+}, and Mg^{2+}. The Zn^{2+}-dependent activity assay of the most active sequence showed that the DNAzyme possesses an apparent Zn^{2+}-binding dissociation constant of 234 μM and that its activity increases with increasing temperatures from 50–90°C. A fit of the Arrhenius plot data gave E_a = 15.3 kcal mol^{-1}. Surprisingly, the selected Zn^{2+}-dependent DNAzymes showed only a modest (~3-fold) activity enhancement over the background rate of cleavage of random sequences containing a single embedded ribonucleotide within an otherwise DNA oligonucleotide. The result is attributable to the ability of DNA to sustain cleavage activity at high temperature with minimal secondary structure when Zn^{2+} is present. Since this effect is highly specific for Zn^{2+}, this metal ion may play a special role in molecular evolution of nucleic acids at high temperature.

Key words: DNAzymes — Deoxyribozymes — Catalytic DNA — Ribozymes — High temperature — RNA hydrolysis — *In vitro* selection — Metal ions

Introduction

Isolation and characterization of thermophilic and hyperthermophilic enzymes is an important research endeavor that can result in both fundamental insight into molecular evolution processes and practical use of these enzymes in biotechnological applications. While earlier efforts have focused mainly on protein enzymes, thermophilic RNAzymes (ribozymes), either purified from thermophilic organisms or selected from *in vitro* evolution, have recently received much attention. For example, thermal denaturation of L21 Sca I, a group I RNAzyme from *Tetrahymena thermophila*, showed that tertiary contacts are disrupted at lower temperatures than secondary structural elements (Banerjee et al. 1993). Studies on a group II intron from *Azotobacter vinelandii* (Adamidi et al. 2003) and on group I introns from hyperthermophilic bacteria of the genus *Thermatoga* (Nesbø and Doolittle 2003) have suggested that increased G+C content plays a role in supporting activity at elevated temperatures. The thermostability of a group I intron from *Azoarcus sp. BH72* has been attributed to the presence of secondary structural motifs that interact strongly with GAAA tetraloops, in addition to the elevated G+C content of the intron (Tanner and Cech 1996). Folding studies with the catalytic portion of the RNase P ribozyme from *Thermus thermophilus* suggest that a less ordered folding intermediate leads to increased stability by adoption of a higher degree of structure in the final folding step, providing a greater thermodynamic impetus for adopting a native configuration (Fang et al. 2001). In addition, step-by-step conversion of mesophilic RNase P from *Bacillus subtilis* to thermophilic variants from *Bacillus stearothermophilus* has been carried out (Fang et al. 2003). The increased structural stability was attributed to increased cooperativity of RNA folding. Hammerhead RNAzymes have also been engineered with increased ter-

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tiary contacts, which lead to increasingly stable tertiary structures and preserve activity up to 80°C (Saksmerprome et al. 2004). *In vitro* selection using the P4-P6 domain of the *T. thermophila* group I intron has also led to RNAs with increased tertiary structural stability, which is attributed to a higher degree of secondary structure (Juneau and Cech 1999). Finally, a variant of the *T. thermophila* L-21 Sca RNAzyme with increased thermostability and activity at temperatures as high as 65°C was obtained through *in vitro* selection (Guo and Cech 2002). Nine mutations contributed to an overall increase in stability of the tertiary structure. From these studies, factors contributing to stabilization and activation of enzymes at elevated temperatures have been elucidated. While protein enzymes from thermophilic organisms achieve stable structures supporting enzymatic activity at high temperature by increasing salt bridges, hydrogen bonds, metal binding, and the compactness of the buried hydrophobic core (Vieille and Zeikus 2001), thermophilic organisms increase RNA stability through increased G + C content (Galtier and Lobry 1997), post-transcriptional modifications (Kowalak et al. 1994; McCloskey et al. 2001), and protein interactions (Brown et al. 1993; Paul et al. 2001).

Previous studies on DNA mini-hairpins showed that it is possible to select DNA with extremely stable structures at high temperatures. For example, such DNA mini-hairpins have been evaluated by *in vitro* selection studies as well as on the basis of structure and nuclease resistance. Studies on the mini hairpins 5′-CGCGAAGCG-3′ and 5′-GCGAAGC-3′ have shown extremely high T_m values of 76.5 and 88.5°C, respectively (Hirao et al. 1989, 1992). Structural studies have attributed the remarkable stability of the small 5′-GCGAAGC-3′ motif to efficient base stacking in the helix and a shear GA base pair at the helix turn (Hirao et al. 1994). *In vitro* selection studies have been used to screen all possible sequences of tri- and tetraloop DNA mini-hairpins for those most resistant to thermal denaturation (Nakano et al. 2002). The identity of the closing base pair was found to have a pronounced effect on the stability of the hairpin structure. In addition, DNA stability studies on small hairpins indicate a greater degree of cooperativity relative to RNA and a lesser degree of permissible sequence variation or mutation in nucleotides involved in these tight interactions (Moody and Bevilacqua 2003a, b).

DNAzymes (deoxyribozymes or catalytic DNA) are a relatively new member of the enzyme family (Breaker and Joyce 1994; Breaker 1997a; Sen and Geyer 1998; Li and Breaker 1999a; Wilson and Szostak 1999; Breaker 2000; Lu 2002; Joyce 2004). Although not observed in nature, DNAzymes with diverse activities have been isolated through *in vitro* selection (Ellington and Szostak 1990; Gold et al. 1995; Osborne and Ellington 1997; Breaker 1997b; Joyce 1999). However, unlike proteins and RNAzymes mentioned above, DNAzymes with activities at elevated temperatures have not been reported. We believe isolation and characterization of such DNAzymes are important for several reasons. First, selecting for DNAzymes with activity at high temperature will test the ability of DNA in forming the sufficiently stable DNAzyme secondary and tertiary structures required to support activity at elevated temperatures. Second, comparing and contrasting structural and functional properties of active DNAzymes at high temperatures with those of thermophilic protein and RNAzymes can provide important understanding of factors contributing to stabilization and activation of all three classes of enzymes. In addition, comparison of the structural and functional characteristics of high-temperature DNAzymes may prove important in investigating whether sequences selected from a random pool at high temperature can achieve similar rate enhancements seen with low temperature DNAzymes. Finally, the increased resistance of DNA to hydrolytic and nuclease degradation relative to proteins and RNAzymes makes DNAzymes attractive candidates for biosensor and therapeutic applications (Cuenoud and Szostak 1995; Breaker 2000; Sun et al. 2000; Lu 2002; Achenbach et al. 2004; Nutiu and Li 2004).

In selecting DNAzymes with activities at high temperature, we decided to focus on DNAzymes with metal ion-dependent cleavage activities. DNAzymes that are highly specific for Pb^{2+} (Breaker and Joyce 1994; Li and Lu 2000), Cu^{2+} (Cuenoud and Szostak 1995; Carmi et al. 1996; Wang et al. 2002), Zn^{2+} (Cuenoud and Szostak 1995; Santoro et al. 2000), and Co^{2+} (Bruesehoff et al. 2002; Mei et al. 2003) have been obtained through *in vitro* selection. In addition, both DNA and RNA aptamers for Zn^{2+} have also been isolated from selection-based approaches (Ciesiolka et al. 1995; Ciesiolka and Yarus 1996; Kawakami et al. 2000). Since *in vitro* selection for metal-dependent sequences has yielded these highly specific constructs with well-defined motifs, we sought to carry out similar selections at high temperature to determine if DNAzymes can be obtained with stable secondary structures at these elevated temperatures and to ascertain what specific contribution metal ions make to the stabilization of secondary and tertiary structures. In addition, structural and functional relationships may exist between DNAzymes that are active at different temperatures. The high metal specificity of DNAzymes has also been exploited for biotechnology applications such as fluorescent (Li and Lu 2000; Lu et al. 2003; Liu and Lu 2003b; Mei et al. 2003; Nutiu and Li 2004) and colorimetric (Liu and Lu 2003a, 2004a, b; Nutiu and Li 2004) sensing of metal and small molecule analytes

(Lu 2002; Nutiu and Li 2004). Obtaining active DNAzymes at higher temperatures may expand the range of possible applications.

Here we report the *in vitro* selection of Zn^{2+}-dependent DNAzymes at 90°C with trans-esterification activity toward a single riboadenosine embedded in a DNA strand. Metal ion selectivity, Zn^{2+}-dependence, and temperature-dependent activity of the most active sequence were investigated. Our results indicate that Zn^{2+} preferentially hydrolyzes RNA linkages at high temperatures and that *in vitro* selection can result in only modest rate enhancement at these temperatures. The implications of these results in molecular evolution and the specificity of the metal assisted RNA hydrolysis reaction for Zn^{2+} at high temperatures are discussed.

Materials and Methods

Reagents and Oligonucleotides

All metal salts used for buffer and stock solutions were Puratronic grade (99.999% pure) and purchased from Alfa-Aesar unless otherwise indicated. All buffers and electrophoresis reagents were purchased from Fisher Scientific or Sigma-Aldrich. Buffers used for *in vitro* selection and kinetic assays were treated overnight with the chelating resin, Chelex 100, and stored in polypropylene vessels previously treated with 10% HNO_3. Reaction buffer used for *in vitro* selection and kinetic assays was 500 mM NaCl and 50 mM HEPES (pH 7.0). All DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. (IDT) and used without further purification. The sequences of the PCR primers P1, P2, and P3, along with the template used for *in vitro* selection are shown in Figure 1A. The biotinylated derivatives of primers P1 and P3, P1b and P3b, were used for immobilization of DNA on a Neutravidin functionalized resin (Pierce). Primer P4 contains an 18-carbon PEG spacer to facilitate gel purification of PCR products (Williams and Bartel 1995). The DNA sequences used for sequence dependence studies are shown in Figure 1B. For steps requiring radiolabeling of oligonucleotides, T4 kinase (Invitrogen) and [γ-^{32}P]-ATP (Amersham) were used.

In Vitro Selection

The *in vitro* selection method for isolation of sequences cleaving a single riboadenosine nucleotide (rA) in an otherwise all DNA strand was adapted from a previously described method (Li et al. 2000). The random pool was generated by template directed extension of 20 pmol of a DNA template followed by PCR amplification. The template used in the pool preparation contained a 40-nucleotide random region flanked by two conserved primer binding regions to allow for PCR amplification using primers P1 and P2 (Fig. 1A). In the random pool and subsequent rounds of selection, a single riboadenosine, which was incorporated using primer P3, served as the cleavage site. The DNA population was then immobilized on a Neutravidin column by using a 5′-biotinylated version of P3. The stability of the Neutravidin biotin interaction was verified by heating biotinylated oligonucleotides immobilized on a Neutravidin column at 90°C for 60 min in a water bath. No DNA eluent was detected after the heating step as determined by agarose gel electrophoresis.

In vitro selection was performed by first immobilizing the DNA population containing the rA on a Neutravidin column, washing the unbound strands from the column, and eluting the all DNA strand using dilute NaOH. The immobilized rA-containing DNA pool was then neutralized with reaction buffer and incubated at 90°C for 3 min prior to the start of the cleavage reaction. The reaction was initiated by addition of $ZnCl_2$ that had been incubated at 90°C for 3 min. The temperature was maintained at 90°C for the remainder of the reaction. Zinc solutions were prepared at twice the final concentration in the 500 mM NaCl and 50 mM (pH 7.0) HEPES reaction buffer. Over the course of the selection, the stringency of selection conditions was increased from round 1 through round 15 by decreasing the reaction time from 60 min to 0.5 min and the $ZnCl_2$ concentration from 1 mM to 0.1 μM. The eluted DNA strands were amplified by two successive PCR reactions for 10–20 cycles. The first reaction contained 40 pmol each of primers P1 and P2. The second reaction contained 40 pmol each of primers P1 and P3. The PCR product from the second PCR reaction was used in the subsequent round of selection.

A
P1 5′-GTGCCAAGCTTACCG-3′
P2 5′-CTGCAGAATTCTAATACGACTCACTATAGGAAGAGATGGCGAC-3′
P3 5′-GGGACGAATTCTAATACGACTCCTATrA-3′
P4 5′-AACAACAACAAC-C_{18} spacer-GTGCCAAGCTTACCG-3′
N_{40} *Template* 5′-GTGCCAAGCTTACCGTCAC-N_{40}-GAGATCTCG
CCATCTCTTCCTATAGGAGTCGTATTAG-3′

B
P3TA 5′-GGGACGAATTCTAATACGACTCCTATrATAATAATAAT-3′
N_5rAN_5 5′-NNNNNrANNNNN-3′

Fig. 1. **A** PCR primers and random pool template, "N_{40}" represents the 40 nucleotide random region, which is flanked by conserved PCR primer binding regions. **B** The sequences of the P3TA and N_5rAN_5 random oligonucleotides.

Kinetic Assays

Intramolecular or *cis*-cleavage of the isolated DNAzymes after different rounds of selection was monitored by a radioactive assay (Brown et al. 2003). For assays using oligonucleotides purchased from IDT, the concentration of DNA was 1 nM. Buffered divalent metal ion solutions were prepared at twice the reaction concentration of both divalent metal ion and buffer. The final buffer and monovalent metal concentrations were 50 mM HEPES (pH 7.0) and 500 mM NaCl, respectively. For reactions carried out at temperatures ranging from 50 to 99°C, samples were heated on a PTC-100 thermocycler (MJ Research, Inc.). Prior to the start of the reaction, the rA containing DNA strands and metal solutions were incubated for 3 min at 90°C or at the temperature to be examined. Each assay was initiated by addition of the buffered metal ion solution. Final divalent metal concentrations for each reaction were 500 μM unless otherwise indicated. At various time intervals, the reaction was quenched with stop buffer containing 8 M urea, 50 mM EDTA, 90 mM Tris, 90 mM boric acid, 0.05% xylene cyanol, and 0.05% bromophenol blue. The cleavage product and unreacted DNA strands were separated on a 20% polyacrylamide gel and quantitated using a Storm 840 Phosphorimager (Molecular Dynamics). Pseudo-first order rate constants were determined by fitting an equation of the form

$$y = y_0 + a(1 - e^{-kt})$$

where y is percent product as a function of time t, y_0 is the background product at time $t = 0$, a is the fraction cleaved at time $t = \infty$, and k is the observed rate constant. The percent product was calculated at time t using the following relation

$$y = 100 \times \frac{\text{amount of product}}{\text{amount of unreacted} + \text{amount of product}}$$

A single trial was performed for the kinetic characterization of individual sequences, or clones, obtained from sequencing of the

round 11 population. Reported rate constants for clone 34 and random oligonucleotides are the average of at least two independent experiments, with the exception of the 60°C rate constant for clone 34, which was performed once. In some assays containing Zn^{2+} and Pb^{2+}, the total radioactivity decreased from 50 to 95% of the initial intensity. This decrease, however, does not noticeably effect the rate because similar behavior was observed for both cleaved and uncleaved DNA strands.

An Arrhenius plot was constructed for variable temperature data. Data was fit to an equation of the form

$$ln(k_{obs}) = lnA - E_a/RT$$

where E_a is the Arrhenius activation energy and A the frequency factor. Thermodynamic parameters were calculated from the application of transition state theory to the Arrhenius data using the equations

$$\Delta H^{\ddagger} = E_a - RT$$

$$\Delta S^{\ddagger} = Rln(k) - Rln\frac{ek_BT}{h} + \frac{E}{T}$$

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$$

where R is the gas constant, k_B is the Boltzmann constant, and h is Planck's constant (Jencks 1969).

Results and Discussion

In Vitro Selection

In vitro selection experiments were carried out to isolate self-cleaving DNAzymes with activity at 90°C. We chose Zn^{2+} because it is a redox-inactive Lewis acid that has been shown to be effective in catalyzing hydrolytic reactions in protein and DNA/RNA-zymes. Fifteen rounds of selection were carried out to isolate high temperature DNAzymes. The concentration of $ZnCl_2$ and time of reaction at the beginning of the selection were 1 mM and 60 min, respectively. In order to increase the likelihood of isolating the most efficient DNAzymes at high temperature, the stringency of the selection conditions was increased over 15 rounds by decreasing the $ZnCl_2$ concentration to 0.1 μM and the reaction time to 30 s. The round 11 population was the most active with $k_{obs} = 0.0263\ min^{-1}$ and a cleavage efficiency of 96.2% after 120 min.

Cloning and sequencing of round 11 resulted in 47 unique sequences (Fig. 2). Kinetic characterization of 24 of these sequences, yielded k_{obs} values ranging from 9.49×10^{-3} to $4.83 \times 10^{-2}\ min^{-1}$ (Table 1). These values correspond to a 1200- to 6200-fold rate enhancement over the Zn^{2+}-dependent RNA cleavage rate of $7.8 \times 10^{-6}\ min^{-1}$ as reported for dinucleotides under similar conditions (Kuusela and Lonnberg 1993). Attempts were made to group the sequences based on secondary structural motifs. However, no secondary structural features were predicted by mfold (Zuker 2003) at temperatures above 82°C.

```
6   CATCTCTAACTAGTACAGGTTCTATACGATATTTTCTGTATCCATG---GTGACG
7   CATCTCGATTGCAAGGATTATAAGAGCTCCAGCCAGATCCTCATC----G-GACG
9   CATCTCCGAGTAAATCAATGACCTTCATCTCCCTAAACACAC-------GTGACG
10  CATCTCTTGTCAAGTAAAGCCTGCCGGATTCAGCGGTTGTTCGTGT---GT-ACG
13  CATCTCCACTCCAACGGGGATTATGACATAGTCCCACTATGGTGA----GTG-CG
16  CATCTCAAATCGACCAATGCCATTAAGCTCCTATGTGTTCTTACTA---GTGACG
17  CATCTCGGCGAGCAATATGTTCTTGGACTCATTCTATTGACTATTG---GT-ACG
19  CATCTCGATCATACACAACCCTTAGGTTGAACCACATTTCCAGCTT---GTGACG
23  CATCTCCATGTGTCAACTCAAGGTTTCTACTTCTTCTGTCCGACAA---GTGACG
25  CATCTCCCAATTACGGAATAAGTGAAATGTCTTAGACCTACTATT----GTGACG
26  CATCTCAAGTCCAACCATCCTCAATAGTGCGGGATTAGGGCACCGT---GTGACG
30  CATCTCGTGCGACAACTCGAGACGCTTTATGCTGTTTAAATTTTCC---GTGACG
31  CAGCTCCGCATGTATCCCAGAGACTTACTGGCCTGATGCTAGCGGT---GTGACG
32  CATCTCGTATGCTGATGCTGGTGCACATGGCGTGACTGATAATCCG---GTGACG
33  CATCTCATTTACTGCTACCTGTTACTGAGCCACAATTGCCCTTACATTAGT-ACG
34  CATCTCTACAGATAGATCAAGACGTCTGGTAGCCTTGCTGTTGGATG--GT-ACG
40  CATCTCTGTTCGCCTCACCCGCTTCGCCCAACTCGCACCTTTATTA---GTGACG
42  CATCTCGCACGTCGTTTCTATTTAGGAAAGAATTAACCTAGGCCTG---GT-ACG
43  CATCTCAAGTAATAAATCGACGACAGATTGGTTTTTTATACCCACG---GT-ACG
44  CATCTCAACGTTGCGCTACGCGATTTGTAGTTGCCTTGCCTTGGTT---GTGACG
46  CATCTCCCGACTGGAACCATTGGTACCCTTTAGCAAGGAGCATAAT---GTGACG
47  CATCTCTCTGGTTGCTGATTCGTATGATTGTCTTAACTACAGGAGC---GTGACG
48  CATCT-TGCATGCCCCAAACACATTCAACACTGCTAAATTGCATT----GTGACG
50  CATCTCGAACAATGCTCATGGTTTGATGACTGTTGATTGTGCGCTT---GT-ACG
51  CATCTCGAGAGGAGTGGATATACCTTGGATTTAGGGCTATCTACCC---GT-ACG
55  CATCTCTGCTTCGCAGTAGGCCAATTATCTCATTCTACCTGCTTGTC--GTGACG
57  CATCTCGTTAACGTGTTGAGCGGACCGAGTGATCTGATATTTCACC---GTGACG
63  CAGC-CATAGTTCTACCAGCGGTTCGAAATAGTGAAGTGTTC-------GTGACT
65  CATCTCCTACCTCCACATTGAGCCGAATTGTCGCCTATTGATACGG---GT-ACG
66  CATCTCAGTATACACGTCGTGATGAACGATAGATGCTTTTACCTNCG--GT-ACG
67  CATCTCAGCATCGCTAGCCCACTACGGGCGCCCGCGCGGCCAATTG---GTGACG
73  CATCTCGATAAAGGTGATTTTAGACTACACTGAGTTAGCTAATCTC---GTGACG
74  CATCTCAGTAAGTGATAATCAAATCGCCCACGTAATACCTNCCATGT--GTGACG
75  CATCTCGAGCCTTAGCCAGTCGGCCCGTCTCGCTCGGATCTCGTCC---GT-ACG
76  CATCTCATTAGCGTACACATTTCATCTTACGACCCTTGTTTACCCC---GT-ACG
79  CATCTCGTGTCTGGTAACGTACACCGTTAATCTAGATCCTACTGG----GT--CG
81  CATCTCCTGGCCCAACGGCGTGAAATCTAGCTTGCCTTTACTTATA---GTGACG
83  CATCTCCCCACAACACTCGATTAAANCCGGGTCTAGTTATCGCGGT---GTGTCG
86  CATCTCGGATCGGAGACGTTCTTATCAATTCACTTACACGGCCGA----GTGGCG
87  CATCTCGGGCCACATAACTGATCCTTAAGGCCTATGAGATTGTT-----GTGACG
88  CACCTCGAAGACCCGCGTTGCACCTGATCAGTTGGGGCCATCCTT----GTGACG
89  CATCTCTACTGAGTTACTCCCACGGTAGCCTTCATTTGGATTGGTC---GTGACG
90  CATCTCCGAAGTACGTTTGTCGTTTGTTAGTTAACGAACGGGGCTG---GTGACG
91  CATCTCGGGACCGTAGACCACGTGCGGAAGGTGGTCACGGCTTGCT---GT-ACG
92  CATCTCCCTTAGATCGGTTTATTCTGGTCCCAATTCTCTCAGGTTT---GTAACG
93  CATCT-GAAGTCGATAGNNTCTCGACTATAGATGANNAATGGTGG----GT-ACG
95  CATCTCAATTAGCTAGTATACAGGTGAGCTATACAGGATGTACCCC---GT-ACG
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Fig. 2. Sequence alignment of clones from round 11. The clone number is shown at the left with the 3′ and 5′ conserved regions highlighted in gray.

The most active sequences were compared with Pb^{2+} (Li et al. 2000) and Co^{2+}-dependent (Bruesehoff et al. 2002) DNAzyme motifs that were selected at ambient temperature but under otherwise similar conditions. No sequence or secondary structural similarities were apparent. The G + C content of the clone 34 DNAzyme was 48%, equal to that of the DNAzymes obtained at lower temperatures. The prediction of minimal secondary structure at temperatures greater than 82°C, although not completely unexpected, is surprising because a number of small DNA hairpin motifs have been reported that melt at temperatures as high as 88.5°C (Hirao et al. 1989, 1992; Nakano et al. 2002). The sequences of the most active clones were also analyzed for the occurrence of these stable stemloop motifs. Three such motifs were found in clones 6, 43, and 50. These structures, however, were not predicted using the mfold program, suggesting that other stable secondary structures may exist that are not predicted by the current mfold algorithm and, therefore, remain to be determined experimentally. A stable stemloop motif was also found in the low temperature Co^{2+}-dependent DNAzyme, but this structural feature was also not predicted by mfold.

Table 1. Kinetic characterization of clones from round 11

Clone	Rate ($\times 10^{-2}$ min^{-1})	Maximum cleavage (%)
76	1.62	75.0
9	2.01	75.1
13	1.06	63.8
16	1.60	79.0
25	1.41	73.2
26	1.25	75.9
30	1.72	66.9
31	1.36	85.2
34	2.43	89.1
42	1.48	72.8
43	4.83	67.4
46	1.17	68.2
47	1.03	65.5
50	1.90	76.1
55	1.95	77.2
65	1.81	75.3
66	1.47	67.1
73	1.59	79.5
74	1.63	67.6
87	1.65	80.3
90	1.61	53.4
91	0.929	53.3

Assays were performed at 90°C with 500 μM Zn^{2+} in 500 mM NaCl, 50mM HEPES (pH 7.0). Cleavage efficiency is reported for reactions heated for 120 min.

The lack of predicted secondary structure at 90°C may have been observed for the following reasons. First, the nearest-neighbor parameters used by the mfold program may not be entirely predictive at such a temperature, and improved parameters for use at elevated temperatures may be needed. As noted above, mfold did not predict formation of the stable tetraloop motifs (Hirao et al 1989, 1992; Nakano et al. 2002) for any of the three occurrences in the round 11 sequences. Second, relatively little predicted secondary structure may indicate that tertiary structural interactions in DNAzymes influence the type of secondary structure that is selected. This hypothesis is supported by recent studies indicating that tertiary interactions in RNAzymes stabilize an active conformation thereby facilitating activity at higher temperatures (Saksmerprome et al. 2004) and decreased divalent metal ion concentrations (Khvorova et al. 2003; Canny et al. 2004; Saksmerprome et al, 2004; Osborne et al. 2005). In addition, these interactions have been shown to increase the cleavage efficiency (Osborne et al. 2005). In selections carried out from random populations at higher temperatures, the absence of tertiary contacts, which stabilize an active fold, may limit both the amount and complexity of secondary structures that are selected. Finally, the sequences selected may be able to sustain cleavage activity at high temperature with minimal secondary structure. This third proposal is supported by our results described here.

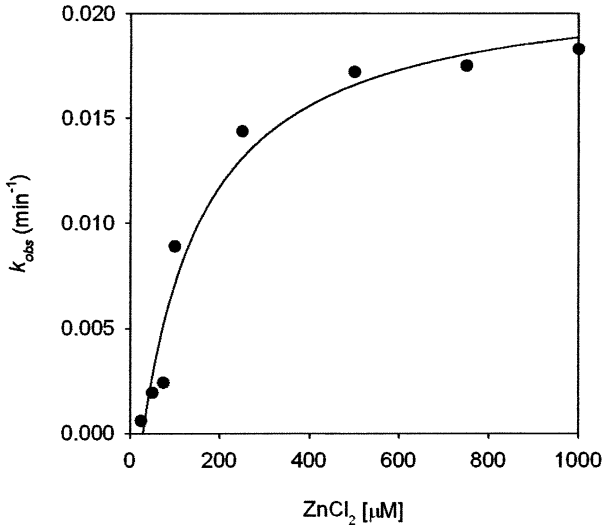

Fig. 3. The Zn^{2+} binding curve measured for clone 34 at 90°C. The K_d was determined to be 234 μM and k_{max} to be 2.43×10^{-2} min^{-1}.

Zn^{2+}-Dependent Cleavage of Clone 34

From the round 11 sequences clone 34 was shown to be the most active and efficient individual with an average $k_{obs} = 0.0201$ min^{-1} and an average cleavage efficiency of 90.1%. Reselection based on clone 34 showed no increase in activity relative to the parent sequence. Therefore, this sequence was selected for further study because it showed a relatively high k_{obs} as well as high degree of cleavage efficiency. The Zn^{2+} concentration dependence of clone 34 was studied at 90°C (Fig. 3), resulting in $K_d = 234$ μM. In the absence of Zn^{2+}, no cleavage at the embedded rA is observed after 48 h.

Temperature-Dependent Cleavage of Clone 34

Temperature dependence studies indicate that clone 34 is much more active at high temperatures (Fig. 4A). A fit of the Arrhenius plot data (Fig. 4B) gave an Arrhenius activation energy of 15.3 kcal mol^{-1}. By applying transition state theory to the Arrhenius plot data, the thermodynamic constraints of $\Delta H^{\ddagger} = 14.5$ kcal mol^{-1}, $\Delta S^{\ddagger} = -26.7$ eu, and $\Delta G^{\ddagger} = 16.9$ kcal mol^{-1} were determined. These parameters are consistent with the range of values reported from temperature-dependence studies of hammerhead RNAzymes (Takagi and Taira 1995; Jankowsky and Schwenzer 1996; Scarabino and Tocchini-Valentini 1996; Baidya and Uhlenbeck 1997; Hammann et al. 1997; Peracchi 1999). The negative entropy value differs somewhat from values reported for 8-17 DNAzymes (Bonaccio et al. 2004) and may reflect the greater difficulty of binding and correctly orienting of Zn^{2+} ions in this

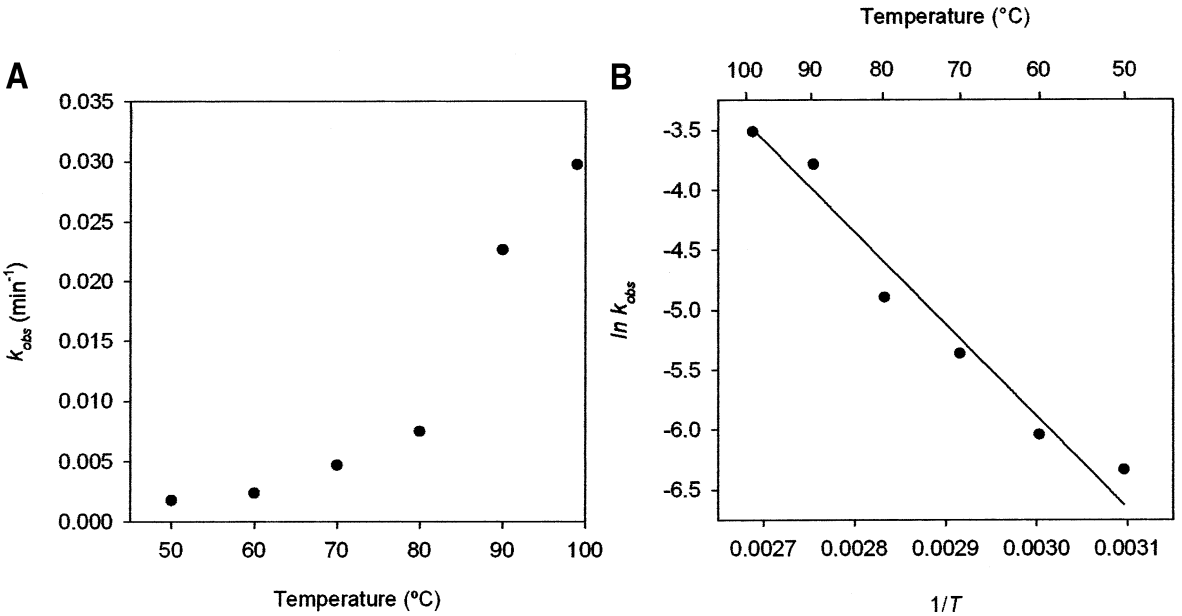

Fig. 4. **A** Temperature dependence of Zn^{2+}-dependent cleavage of clone 34. **B** The Arrhenius plot based on temperature dependence data. Fitting the data to a linear regression gave E_a = 15.3 kcal mol^{-1}.

DNAzyme system. The decrease in rate observed at lower temperatures may be due to the formation of inactive secondary or tertiary structures, which impede access of Zn^{2+} to the cleavage site or to a change in the orientation of the Zn^{2+} ligands preventing the Zn^{2+} ion from being correctly positioned to effect cleavage (Soukup and Breaker 1999).

Reactivity of Random Sequences with Zn^{2+} at 90°C

The observed rate constant for the round 11 (k_{obs} = 0.0263 min^{-1}) is ~3400 times faster than the Zn^{2+}-dependent RNA cleavage rate for dinucleotides (Kuusela and Lonnberg 1993). Only a modest increase in activity, however, was observed over the course of the selection. Therefore, we decided to test Zn^{2+}-dependent cleavage of random rA containing oligonucleotides at high temperature using the N_5rAN_5 sequence (Fig. 5). The N_5rAN_5 sequence consists of 5 random nucleotides flanking the riboadenosine on both sides. This pool of sequences was found to support a modest rate of cleavage in the presence of 500 μM Zn^{2+} at 90°C, with an average observed rate of 5.93 × 10^{-3} mm^{-1}, which is 3.4 times slower than that of clone 34, with a cleavage efficiency as high as 43.4% after 2 h. In order to further rule out any contribution from flanking DNA sequences on the metal-dependent cleavage of the RNA linkage, the short oligonucleotide P3TA, a variant of primer P3 that contains a short 5′-TAATAATAAT-3′ run 3′ of the riboadenosine, was assayed for Zn^{2+}-dependent cleavage. Kinetic studies at 90°C showed that this sequence does indeed cleave in the presence

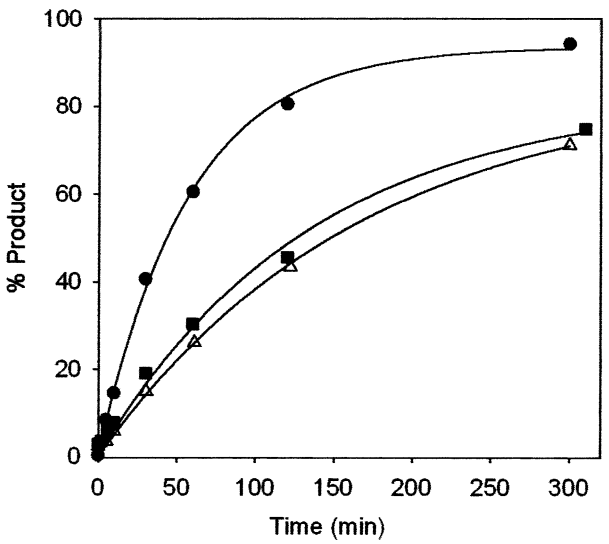

Fig. 5. Kinetic data for (●) clone 34, the (Δ) N_5rAN_5 random sequence, and (■) P3TA at 90°C in the presence of 500 μM Zn^{2+}. Rate constants determined from these individual assays are k_{obs}(clone 34) = 1.77 × 10^{-2}, k_{obs}(N_5rAN_5) = 6.03 × 10^{-3}, and k_{obs}(P3TA) = 7.48 × 10^{-3} min^{-1}. Average values are reported in the text.

of Zn^{2+} with an average k_{obs} = 5.93 × 10^{-3} min^{-1} and showing up to 45.5% cleavage after 2 h (Fig. 5). This rate is again slower than that of clone 34 by a factor of 3.4. Figure 6 shows a typical gel electrophoresis image of the kinetic assays performed on clone 34, P3TA, and N_5rAN_5. The rate enhancement for clone 34 is smaller than expected because the background rate of cleavage of random oligonucleotides was larger than the initial estimate based on the Zn^{2+}-mediated hydrolysis of dinucleotides. This

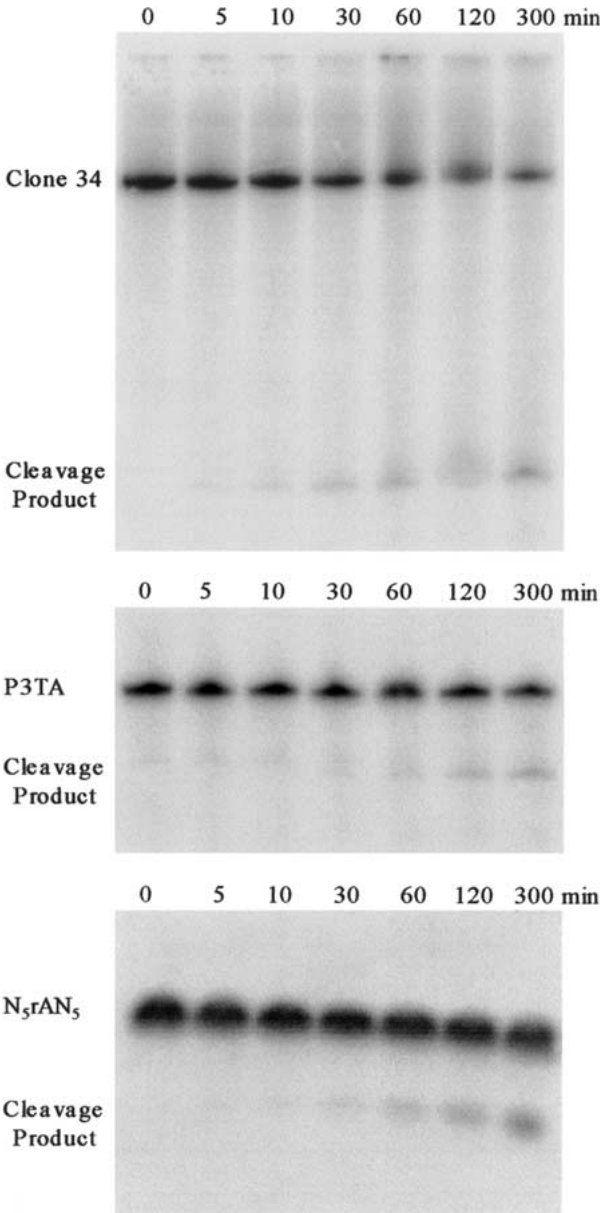

Fig. 6. Gel electrophoresis image of clone 34 (**top**), P3TA (**middle**), and the N_5rAN_5 random sequence (**bottom**) heated at 90°C in the presence of 100 μM Zn^{2+}. The cleavage efficiencies were 21% for clone 34, 10% for P3TA, and 16% for N_5rAN_5 after 120 min.

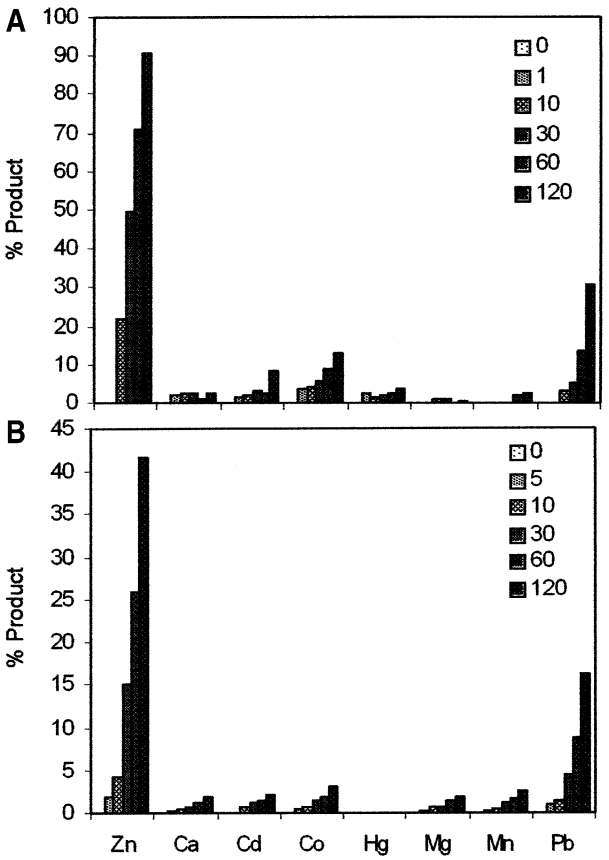

Fig. 7. Metal specificity of cleavage of (**A**) the round 11 population and **(B)** the N_5rAN_5 random sequence. Assays were performed in the presence of 500 μM metal ion at 90°C. Graph legends indicate time of heating in min. Please note that the vertical axes have different scales.

large background rate may preclude observing the large rate enhancements that are routinely observed for DNAzymes selected at lower temperatures. The increase in both rate and efficiency of an already fast reaction, however, indicates that a molecular evolutionary process has selected for a relatively efficient DNAzyme under these extreme conditions.

Metal Ion-Dependent Studies

The modest rate enhancement of Zn^{2+}-dependent DNAzyme from *in vitro* selection raises interesting questions about the likelihood and sequence dependence of RNA hydrolysis by other divalent metals at 90°C. The metal specificity of the round 11 population and the N_5rAN_5 sequence was measured at 90°C for 500 μM Ca^{2+}, Cd^{2+}, Co^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, and Pb^{2+}. Only the metal ions Co^{2+} and Pb^{2+} show appreciable cleavage after 2 h (Fig. 7 A), cleaving 13.0 and 30.7% of the sequences, respectively. The ratio for the Zn/Co efficiency is 7.0, and the Zn/Pb efficiency is 3.0, indicating a high degree of specificity for Zn^{2+}-dependent RNA hydrolysis at 90°C. Similarly, the relative efficiencies of metal-dependent hydrolysis for the N_5rAN_5 sequence, although less efficient than those observed with the round 11 population, also showed a distinct preference for Zn^{2+} (Fig. 7B).

These results indicate that Zn^{2+}-dependent hydrolysis of a single embedded ribonucleotide at high temperatures is a facile reaction and that *in vitro* selection resulted in only a modest increase (~ 3-fold) in DNAzyme activity. This result is surprising, as *in vitro* selection for Zn^{2+}-dependent DNAzymes at room temperature results in much higher rate enhancements (10^6-fold) (Breaker and Joyce 1995; Li et al. 2000). Rate enhancements of this magnitude,

however, may not be expected from DNAzymes selected here for several reasons. At 90°C the background rate was found to be 10^4-fold greater than at 25°C, which limits the rate enhancement considerably, compared with low temperature selections. In addition, the RNAzymes that have been reported with activity at elevated temperatures have been isolated based on previously defined low-temperature RNAzyme motifs. We sought instead to investigate the more difficult scenario of obtaining DNAzymes with increased structural stability from a population of random sequences. Other factors such as sequence identity and length have also been found to affect metal-dependent RNA stability (Butzow and Eichhorn 1971; Kaukinen et al. 2002; Breaker and Joyce 1995; Li et al. 2000; Guo and Cech 2002). Sequence specificity of Zn^{2+}-dependent strand scission was also observed to decrease at high pH (Li and Breaker 1999b; Kaukinen et al. 2002).

While it is tempting to attribute the efficient Zn^{2+} hydrolysis at higher temperature and elevated pH to the pK_a of metal bound water molecules or to the accessibility of the phosphoester linkage to the Zn^{2+} metal ion, the actual explanation may not be quite as straightforward. The high efficiency of Zn^{2+}-dependent cleavage of clone 34 cannot be accounted for solely on the basis of the pK_a of Zn^{2+} bound water ($pK_a = 9.0$), because the DNAzyme shows lower activity and efficiency with Pb^{2+} ($pK_a = 7.8$). In addition, it has recently been proposed that the increased ability of a sequence to sample the inline conformation of the nucleophile for the scissile phosphate leads to an increased rate (Soukup and Breaker 1999). At elevated temperatures, the increased thermal motions of even a relatively unstructured sequence will likely lead to the strand and metal cofactor achieving the correct orientation for metal assisted strand scission more often than at lower temperatures. The potential ligands adjacent to the cleavage site may be sufficient to bind Zn^{2+} to effect cleavage. The N1 and N7 purine bases may both play minor roles as Zn^{2+} ligands (Martin 1985). The scissile phosphate oxyanion (Ikenaga and Inoue 1974) and the 2′-OH (Butzow and Eichhorn 1971) are likely to be the predominant ligands. Another report indicates that when three of the coordination sites of Zn^{2+} are occupied, the sequence dependence is decreased (Mikkola et al. 1999). Although the binding may be more transient at high temperatures, as reflected by the relatively low affinity of clone 34 for Zn^{2+}, the accessibility of the scissile phosphate and the conformational flexibility may promote the Zn^{2+}-dependent cleavage even though a precise metal binding pocket may not be formed as at room temperature. The activity and specificity of the DNAzymes reported here suggests that incorporating stable secondary structure into the pool design may lead to improvements in rate enhancement for high temperature selections and that Zn^{2+} may play an important role in high temperature molecular evolutionary processes involving nucleic acids.

Conclusions

In summary, Zn^{2+}-dependent DNAzymes with RNA-cleaving activities have been obtained through *in vitro* selection at 90°C, the highest reported temperature at which such studies have been carried out. The selected DNAzymes display a number of unique sequences, from which the most active clone was characterized. The most active clones showed little sequence similarity when compared with other metal-dependent DNAzymes. The DNAzymes showed high specificity for Zn^{2+}, but only modest rate enhancement over the background rate of cleavage for random chimeric sequences containing a single ribonucleotide. These results indicate the need for further studies to better understand the relationships between the structural and functional characteristics of DNAzymes at high temperatures in order to facilitate the design and directed evolution of more efficient nucleic acids enzymes. The ability of Zn^{2+} to support DNA cleavage activity at high temperature with minimal secondary structure also suggests that this metal may play a special role in molecular evolution of nucleic acids at high temperature.

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