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High affinity DNAzyme-based ligands for transition metal cations – a prototype sensor for Hg^{2+}

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Inspired by recent interest in DNAzymes as transition metal ion sensors, a survey of the effects of various transition metals on the intramolecular cleavage rate of an imidazole modified, M^{2+}-independent, self-cleaving "9_{25}–11" DNA is reported. In particular, 9_{25}–11 activity was strongly inhibited by Hg^{2+} ($K_d^{APP} = 110 \pm 9$ nM). It is postulated that the affinity and selectivity of 9_{25}–11 for Hg^{2+} stems from the fact that this synthetically modified DNAzyme contains imidazoles. This study demonstrates the utility of modified nucleotides in developing DNAzyme sensors for metals ions, especially those for which unmodified nucleic acids might not serve as inherently good ligands.

Introduction

Since the recent advent of DNAzymes (catalytic DNA), DNA has now been found to catalyze many reactions that include ligation,[1–3] phosphorylation,[4] RNA cleavage,[5] oxidative DNA cleavage,[6] porphyrin metallation,[7] and depurination[8] to name a few. Most DNAzymes are metalloenzymes and, quite commonly, the activity for which they have been selected involves cleavage of a complementary oligonucleotide substrate by virtue of a divalent metal cation cofactor. In one case, a DNAzyme was selected to promote oxidative scission of a complementary DNA substrate in the presence of 100 μM copper.[6] In contrast to oxidative cleavage, DNAzymes have also been selected to effectively hydrolyze a ribophosphodiester (RNA) linkage embedded within a complementary substrate, usually in the presence of Mg^{2+} or another divalent metal cation. In some cases, substitution of other metal cations in place of magnesium cation improves the properties of RNA-cleaving DNAzymes, either in terms of increased k_{cat}, decreased $K_{d(metal)}$, or both.[9] Of the common d-block metal cations, Pb^{2+} has been of primordial importance with regards to RNA cleavage as it affords a metal-aqua (or a 2′-alkoxide) species with an especially low pK_a that makes it a particularly impressive catalyst for RNA hydrolysis, even when examined independently of a catalytic nucleic acid scaffold (*i.e.* a DNAzyme or ribozyme).[10] Lead cation was used in the selection of a small self-cleaving RNA (the "leadzyme"),[11] as well as the first RNA-hydrolyzing DNAzyme.[12] Since then, certain DNAzymes that normally use Mg^{2+} can also use Pb^{2+} at significantly lower concentrations without loss of activity. Specifically, the 8–17 DNAzyme can use Pb^{2+} at concentrations as low as 1 μM, to cleave RNA.[13] When a beacon-substrate (*e.g.* derivatized with FRET reporters or nanogold particles) is used, the 8–17 DNAzyme can act as a highly specific sensor for lead.[14,15]

Lead cation, as an environmentally unfriendly analyte for detection by DNAzymes, has been a "stand-alone" in terms of metal cation sensing. In all likelihood, this has to do with its unique ability to a) associate with DNAzymes in place of Mg^{2+} and Mn^{2+} or b) serve as a highly efficient catalyst for RNA hydrolysis, or both. Tailoring DNAzymes for alternate metal cation specificities apart from lead to include other particularly toxic ones such as mercury represents an active avenue of investigation. Various approaches to this end have included selections (negative and positive) from pools of metal cations at different pHs.[16,17] Such approaches are leading to new classes of DNAzymes and self-cleaving DNAs with enhanced specificities for certain metal cations that derive either from direct catalysis by the metal cation (*e.g.* Lewis acid, pK_a perturbation) or potentially from allosteric effects that promote proper catalyst folding.

In order to increase the chemical diversity of metal cation binding sites, additional chemical functionalities such as imidazoles and pyridyls, which are initially bourn on the monomer triphosphates used in a combinatorial selection, have been incorporated into duly modified metallo-DNAzymes. This synthetic effort has lead to the discovery of highly efficient zinc-dependent RNase,[18] copper-dependent Diels–Alderase,[19] and copper-dependent amide synthase[20] activities. These all operate with metal cation concentrations on the order of 10–100 μM M^{2+}. A different approach to improving the specificity for sensing certain metal cations could involve adding synthetic functionality to the substrate. For example, the hammerhead ribozyme was engineered to "sense" Hg^{2+} when the scissile phosphate of the substrate was replaced with a thiophosphate such that rescue could be observed at 500 nM Hg^{2+}.[21]

Apart from this hammerhead study, Hg^{2+}, unlike Pb^{2+}, has not been particularly revealing of any extraordinary activity with respect to catalytic nucleic acids (ribozymes and deoxyribozymes), either in terms of stimulation or inhibition, where either action would identify a paradigm for sensing this very toxic and industrially abundant cation. The indolence of Hg^{2+} in this context is perhaps attributable to the fact that nucleic acids in general and DNA in particular do not provide good ligands for Hg^{2+}. Indeed, the nature of Hg^{2+} association with nucleic acids has been studied with respect to both RNA and DNA as well as on mononucleotides *via* spectral shifts and viscosity studies.[22–26] A reaction of note is the covalent mercuration of the 5 position of pyrimidines, namely at U in RNA, but also at C in both RNA and DNA.[27] Mercuration proceeds at low ionic strength and is suppressed by high salt in general, and by chloride ions in particular. Once formed, this covalent adduct is quite stable and thus not readily described by a dissociation constant. By contrast, several studies have described noncovalent DNA–Hg^{2+} interactions. The purine-N7 has also been described as an effective chelator of mercuric ion. Recent work indicates that the noncovalent interaction of DNA with Hg^{2+} seems to be largely limited to chelation by anionic N3 of T at high pH,[28–30] a protonation state that is thermodynamically disfavored at physiological pH and kinetically disfavored in base-paired duplexes. Apart from these interactions, neither the other nucleobase atoms nor the phosphate is a particularly good ligand for mercuric ions; most of the dissociation constants for Hg^{2+} association with nitrogen and oxygen

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donors on DNA lie in the range of 10^{-5}–10^{-3} M.[31] Given the limited ability for DNA to effectively chelate Hg^{2+}, it is challenging to imagine how DNAzymes (or ribozymes) might be selected to sense Hg^{2+}, and yet perhaps not entirely surprising that Hg^{2+} has not been implicated in terms of nucleic acid catalysis or the inhibition thereof.

We have been interested in developing methodologies that enhance the chemical functionality of DNA and then in identifying activities (binding, catalysis, medicinal) that i) will necessarily depend on these functionalities and, ii) more importantly, will not likely be discovered in combinatorial selections undertaken with unmodified nucleic acids. We began this development by elaborating synthetic and enzymatic methodologies that would allow for the simultaneous incorporation of 8-histaminyl-dATP [8-(2-(4-Imidazolyl)ethylamino)-2′-deoxyriboadenosine triphosphate] and AA-dUTP [5-(3-Aminoallyl)-2′-deoxyribouridine triphosphate] in place of their respective unmodified counterparts dATP and dTTP.[32] Using these triphosphates, we then selected a DNA strand that self-cleaves at an internal RNA linkage in the absence of any divalent metal cation.[33] This self-cleavage reaction likely involves general acid or general base catalysis or both, as well as electrostatic stabilization that derives from synthetically appended imidazoles and cationic amines respectively, and upon which this self-cleaving RNaseA-like activity is obligately dependent. The self-cleaving species is shown in Fig. 1 and forms the basis for the investigation undertaken herein. Because the cleavage proceeds in the absence of a divalent metal cation, no divalent metal cation binding site had necessarily been selected. Instead, the active site is composed of a metal-free environment occupied by imidazoles and amines. Interestingly, metal cations that normally support DNAzyme catalysis of ribophosphodiester hydrolysis, *e.g.* Mg^{2+} and Ca^{2+}, had no effect on this self-cleavage reaction, underscoring the fact that the active site may be passive with respect the presence of certain metal cations, particularly those of the "hard" alkali earths.

In light of this observation, we were intrigued by the possibility that the catalytically important imidazoles and amines might provide fortuitous binding sites for other d-block metal

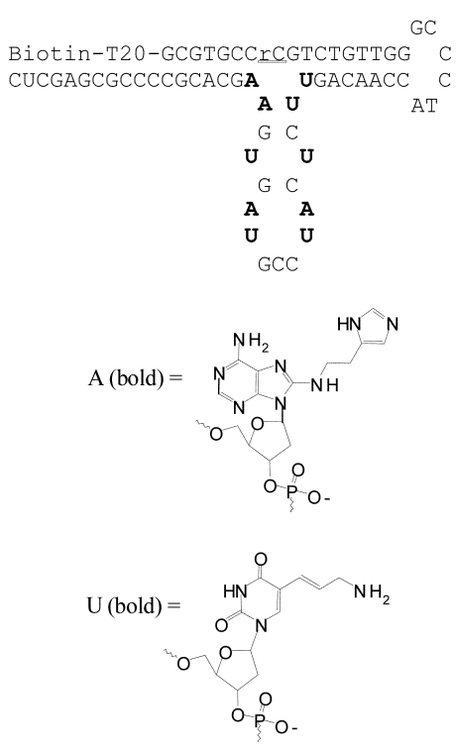

Fig. 1 The self-cleaving species 9_{25}–11 is shown. This consists of one embedded ribophosphodiester linkage, a hairpin loop and a catalytic motif containing 6 aminoallyl-dUs (**U**) and 4 histaminyl-dAs (**A**).

cations that normally exhibit high affinity for nitrogen ligands. In so far as "soft" d-block metal cations might compete for these imidazoles and consequently inhibit cleavage, the inhibitory activity could be characterized in terms of metal specificity, and in terms of affinity. As such, this M^{2+}-independent self-cleaving DNA could then be viewed as a potential sensor provided that the inhibition would be specific to one or but a few metal cations. A series of metal cations were investigated as inhibitors of the self-cleaving activity. This inhibition can be correlated to the binding selectivity and from an analysis of initial rates *vs.* metal ion concentration, a K_d could be calculated. This report suggests that modified nucleotides will enhance the selectivity and the affinity of DNAzyme in terms of metal cation sensing.

Results

Based on the previously reported observation that Cu^{2+}, Ni^{2+}, and Zn^{2+} completely inhibited self-cleavage at 500 μM, we undertook a more comprehensive survey of metal cations that might be expected to display significant affinity for the imidazoles and amines that are involved in catalysis. The metal cations we examined at 10, 30 and 100 μM are: Ag^{+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Ce^{3+}, Ce^{4+}, Co^{2+}, Cu^{2+}, Eu^{3+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, La^{3+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Sm^{3+}, Sr^{2+}, Y^{3+}, Yb^{3+}, and Zn^{2+}. The self-cleaving species was pre-incubated with the metal cations in water for 30 minutes. Cleavage was then initiated by adding an equal volume of "2×" buffer + NaCl to allow for refolding and cleavage. Inhibition of cleavage was assayed based on the extent of cleavage within the first ten minutes (initial rates). Direct addition of the self-cleaving DNA (in water) to a "2×" cleavage solution containing NaCl, buffer, and the metal cation of interest gave qualitatively the same result indicating that the metal cation association was fast relative to either folding or cleavage. Within the concentration range 10–100 μM, Ce^{3+}, Ce^{4+}, Cu^{2+}, Eu^{3+}, Fe^{3+}, Hg^{2+}, La^{3+}, Ni^{2+}, Sm^{3+}, Y^{3+}, Yb^{3+}, Zn^{2+} showed some inhibitory properties (Fig. 2), with the most pronounced effect being observed for Hg^{2+}. Antecedent studies indicated that Hg^{2+} has the greatest binding power for imidazoles; $\log\beta_2$ (K_a) for the bis-imidazole Hg^{2+} chelate ranges from 15 to 21 with increasing pH.[34] It was thus not entirely surprising that of the cations examined, Hg^{2+} would be the most effective inhibitor of a DNAzyme where imidazoles are required for activity.

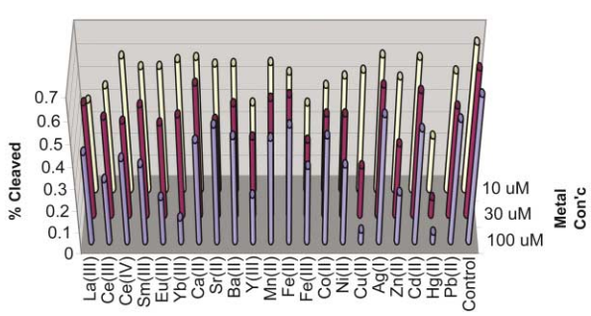

Fig. 2 Survey of the effect of various metals (at 10, 30, and 100 μM) on the self-cleavage activity of 9_{25}–11. All reactions: 25 mM cacodylate (pH 7.5), 200 mM NaCl, and transition metal ion (M^{n+}) at indicated concentrations. Metal survey reactions were quenched after 10 minutes with 15 μL of 500 μM biotin, 25 mM EDTA in formamide. Samples were then resolved by 20% denaturing urea-PAGE.

To begin to characterize this interaction we were mindful of the fact that instead of complexing with the imidazoles, Hg^{2+} could have mercurated the 5-position of C or reacted irreversibly with olefins to give covalent Markovnikov addition products *i.e.* hydroxyalkylmercurials. We wanted to exclude the possibility of hydroxymercuration of the olefinic allylamino functionality (as well as any fortuitous mercuration at the 5 position of cytosine) by demonstrating that the observed

inhibition was readily reversible and thus entirely due to a dissociable metal cation nitrogen interaction that most likely involved the imidazoles. To demonstrate this, the self-cleaving DNA was first poisoned at 30 μM Hg^{2+} where little cleavage could be observed within the first 15 minutes. Nevertheless, subsequent addition of EDTA (25 mM final) or DTT (10 mM final) completely restored the self-cleaving activity. These effects are shown in Fig. 3. In addition, at much longer times, cleavage in the presence of Hg^{2+} proceeded to the full extent (data not shown), further substantiating that the Hg^{2+} interaction is reversible and thus the unbound components (Hg^{2+} and free DNA) are likely to be in rapid equilibrium with the bound species. Moreover, the fact that DTT and EDTA rescued cleavage suggested that the DNA was either properly folded or at least not kinetically trapped in an inactive conformation induced by Hg^{2+} binding.

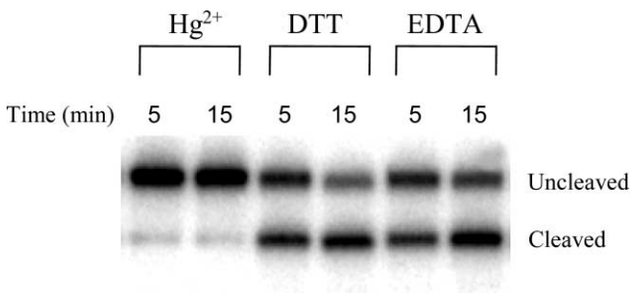

Fig. 3 Rescue experiments illustrating the reversibility of Hg^{2+} inhibition. All lanes: 30 μM Hg^{2+}, 25 mM cacodylate (pH 7.5), 200 mM NaCl. Lanes 3 and 4 rescued with 1/10th volume 100 mM DTT, lanes 5 and 6 rescued with 1/10th volume 250 mM EDTA.

In order to calculate an apparent dissociation constant (K_d^{APP}) for Hg^{2+} binding, a steady-state rate approximation with regard to initial rates was adopted along with three simplifying assumptions that *i*) the Hg^{2+}-bound DNA was totally inactive and that only the free DNA could self-cleave, *ii*) that the binding of only one Hg^{2+} was necessary for inhibition (a Hill plot analysis, *vide infra*, indeed showed this), and *iii*) the cleavage reaction, as measured by initial rates, was taken to be irreversible *i.e.* religation was not operative. Self-cleavage reactions were initiated at several concentrations of Hg^{2+} and the reciprocals of observed cleavage rate constants were plotted *vs*. Hg^{2+} concentration. This reciprocal plot gave a linear relationship with the K_d^{APP} appearing in the slope (see kinetic scheme in methods section). Cleavage activity as a function of Hg^{2+} concentration is shown in Fig. 4, and a direct plot of cleavage rate constant *versus* Hg^{2+} concentration is shown in Fig. 5. The plot of k_{obs}^{-1} *vs*. Hg^{2+} concentration is given in Fig. 6; from the slope of this plot a K_d^{APP} of 110 ± 9 nM was calculated for Hg^{2+}.

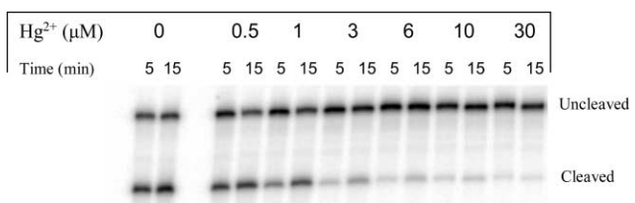

Fig. 4 Hg^{2+} inhibition titration of 9_{25}–11. All reactions: 25 mM cacodylate (pH 7.5), 200 mM NaCl, and Hg^{2+} at indicated concentrations.

To ascertain the number of binding sites for Hg^{2+}, a Hill plot analysis was undertaken as shown in Fig. 7. Because the extent of inhibition is high, the slope of this plot approximates the number of binding sites.[35] With a constant slope near unity, these results show that there is no cooperative interaction, and that in all likelihood, there is only one binding site for Hg^{2+}. If

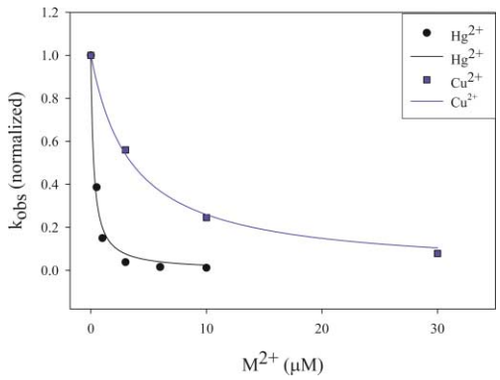

Fig. 5 Plots showing the inhibition of 9_{25}–11 activity as a function of Hg^{2+} or Cu^{2+} concentration – k_{obs} is a relative value that is normalized against the uninhibited rate constant.

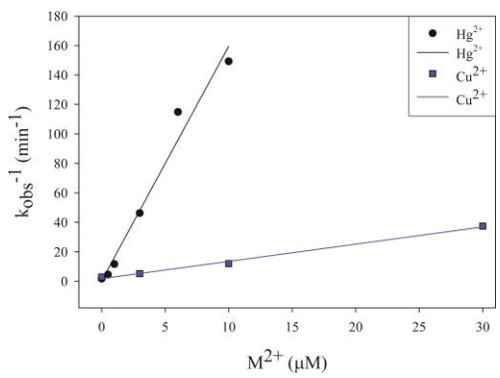

Fig. 6 Plot of k_{obs}^{-1} *vs*. Hg^{2+} or Cu^{2+} concentration (based on a steady state inhibition model). K_d was determined to be 110 ± 9 nM for Hg^{2+} and 2.5 ± 0.1 μM for Cu^{2+} from slope of these plots using eqn. 2 (see kinetic scheme).

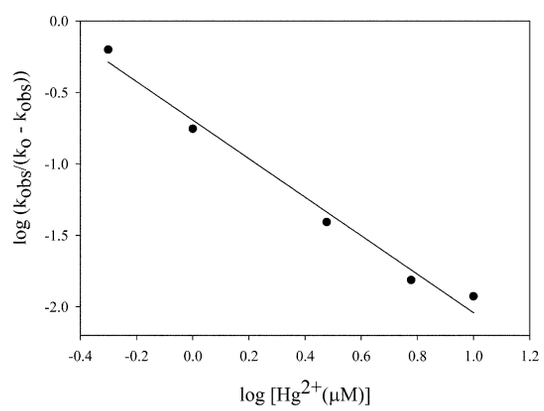

Fig. 7 Hill plot for Hg^{2+} inhibition of 9_{25}–11. The slope of ~1.3 suggests the presence of one mercury binding site.

there were more than one Hg^{2+} binding site, which might still be possible as there are four imidazoles, it remained undetected in this analysis.

Following Hg^{2+}, the next, most inhibitory metal cation found in our survey was Cu^{2+}. Figs 5 and 6 also show the inhibitory effect of Cu^{2+} compared to that of Hg^{2+}. In the same manner as for Hg^{2+}, a K_d^{APP} of 2.5 ± 0.1 μM was obtained for Cu^{2+}. This value suggests that the selectivity for Hg^{2+} over Cu^{2+} approaches a value of 25-fold when calculated from initial rate constants. For use as a sensor, one must consider the effect of a second metal cation that could compete with Hg^{2+} binding to either inhibit self-cleavage (in the case of cupric ion) or prevent Hg^{2+} from binding thus masking the presence of Hg^{2+}. Thus, the effects of both the inhibitory Cu^{2+} and the non-inhibitory Ca^{2+} were tested under conditions where Hg^{2+} could be sensed, but where these two cations would not be expected to exert a pronounced effect on self-cleavage or Hg^{2+} inhibition. Fig. 8

shows the cleavage kinetics for inhibition by 3 μM Hg^{2+} in the presence and absence of 3 μM Cu^{2+} or Ca^{2+}. At this concentration, these spectator cations do not appear to significantly perturb the effect of Hg^{2+} on the observed rate constant for self-cleavage.

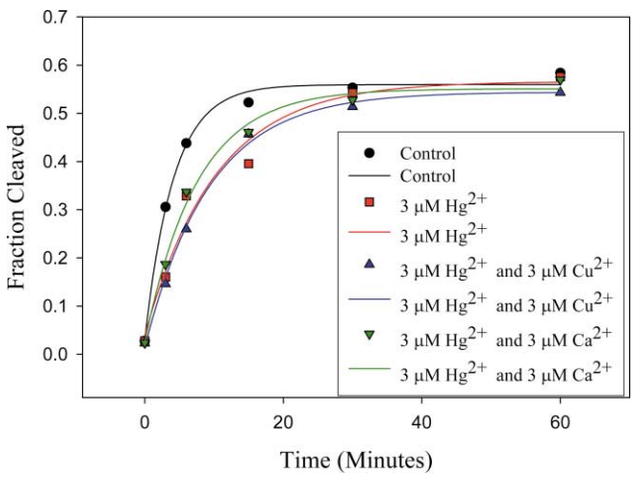

Fig. 8 The effect of two spectator metal ions on the Hg^{2+} inhibition of 9_{25}–11. The rate constants determined are as follows: k(control) = 0.24 ± 0.02 min^{-1}, k(Hg^{2+}) = 0.10 ± 0.03 min^{-1}, k(Hg^{2+} + Cu^{2+}) = 0.11 ± 0.01 min^{-1}, k(Hg^{2+} + Ca^{2+}) = 0.13 ± 0.02 min^{-1}. Inhibition by 3 μM Hg^{2+} is not significantly affected by the presence of 3 μM Cu^{2+} or 3 μM Ca^{2+} (the rate constants agree within the standard errors of the fits).

Discussion and conclusions

The sensing based on self-cleavage herein is neither reversible nor catalytic as with the 8–17-DNAzyme. Nevertheless, others have proposed potential sensors based on the irreversible activity of fluorescently labeled self-cleaving DNAs.[36] Admittedly, one further drawback is that sensing herein is based on inhibition of cleavage rather than stimulation of cleavage. Nevertheless, this could in theory be turned into a positive read-out. For instance, one could envision a chip-based approach that would include fluorescent dyes and/or quenchers appended at either side of the scissile ribose so that the presence of Hg^{2+} could be detected with rapid read-out. In terms of specificity and affinity, this work parallels recent work by Nolan and Lippard, who reported a fluorescent sensor for mercuric ion (fluorescein appended with a bisthioetheraminomethylaniline) that gave a 50% fluorescence enhancement in the presence of 410 nM Hg^{2+}. They also reported high selectivity over other ions with the exception of Cu^{2+}, which effectively competed with Hg^{2+} binding.[37] Along the same lines as theirs, the work herein also demonstrates good selectivity for mercuric ion over other di- and trivalent metal cations (1–2 orders of magnitude), with the exception being that of cupric ion which acted very similarly to inhibit self-cleavage at 25-fold higher concentrations (K_d^{APP} of 2.5 ± 0.1 μM). With a K_d^{APP} of 110 ± 9 nM for Hg^{2+}, this DNAzyme is capable of sensing Hg^{2+} at the level of 2 ppb. Whereas further biophysical and chemical studies will no doubt provide more insight into the structural basis of this inhibition, this work now presents the first case of a submicromolar interaction of a DNAzyme with Hg^{2+}.

It is noteworthy that this DNAzyme was selected for M^{2+}-independent cleavage that occurs by virtue of the synthetically appended imidazoles and amines. This M^{2+}-independence provides for a reaction path whereby inhibitory metal cations, in this case Hg^{2+}, can compete for synthetic functionalities that are involved in catalysis, rather than for the binding site of a different catalytic metal cation. In spite of the well-known affinity of imidazoles for Hg^{2+}, the imidazoles in this self-cleaving DNA sequence convey but a modest (submicromolar) affinity for Hg^{2+}. This apparent affinity, which falls far short of the expected imidazole-Hg^{2+} interaction, may be due to the positioning of the imidazoles that are oriented to favor M^{2+}-independent ribophosphodiester hydrolysis, rather than Hg^{2+} binding. Because no divalent metal cation had been present in this selection, these inhibition results probably describe an upper limit for Hg^{2+}-recognition, *i.e.* ~2 ppb and a starting point for reselection with imidazole modified dNTPs to improve detection.[38] Reselection of self-cleaving imidazole-modified DNAs, with or without other hard metal cations that are commonly used to stabilize DNA structures, but where self-cleavage is sought by the addition of Hg^{2+} should improve the affinity quite dramatically. Such a selection would ensure that metal cation binding could be used to trigger cleavage rather than inhibit it. Following such selection, it will be interesting to see whether Hg^{2+} will bind to the DNAzyme's active site as the metal cation cofactor directly responsible for chemical catalysis, or simply act as an allosteric effector that brings two imidazoles together to staple the DNAzyme into a catalytically competent conformation. This work also suggests an *in vitro* approach to sensing mercury with DNAzymes in contrast to cell-based approaches. Nevertheless, it remains to be seen whether modified DNAzyme sensors will attain the 2 ppt level seen with other *in vivo* cell-based sensors such as those that rely on MerR-reporter gene constructs.[39,40]

In another application of imidazole modified DNA, one might also envision using mercury to mediate the interaction between imidazole modified DNA aptamers and imidazoles on proteins *via* a mercury "bridge" to afford specific recognition of protein surfaces. Indeed, imidazole–mercury bridging for specific protein recognition had been suggested a decade ago in the context of small ligand design and templation.[41] Imidazole-modified rNTPs may also be useful for probing RNA folding if two such nucleotides can be properly placed within a ribozyme such that they afford a specific mercury chelate, which might stabilize conformationally distinct species. Such mercury bridging could be released quickly and selectively by DTT so that refolding may be studied in the presence of Mg^{2+} and Ca^{2+}.

This work demonstrates how synthetically modified dNTPs can deliver new and unusual properties to DNAzymes, and is another example of how synthetic functionalities may considerably enhance the functional repertoire of nucleic acids. Certainly the idea of using synthetically appended dNTPs to select for Hg^{2+} sensors should not be limited to imidazoles. Indeed, thiols should impart affinities of equal if not much higher values. Recently, thiol modified dNTPs have been shown to be compatible with SELEX (systematic evolution of ligands by exponential enrichment) and related combinatorial *in vitro* selection procedures that make use of extraordinarily large libraries of DNA and RNA.[42–44] Thiol-bearing DNAzymes should give rise to even more potent Hg^{2+} sensing DNAzymes provided that the thiols remain reduced if sensing is conducted under aerobic conditions.[45,46]

Materials and methods

The syntheses of 5-aminoallyl-dUTP ($dU^{aa}TP$) and 8-histaminyl-dATP ($dA^{im}TP$) have been described in detail elsewhere.[20] dGTP α-[^{32}P] was obtained from Perkin-Elmer. Avidin magnetic particles and unmodified dNTP's (PCR grade) were obtained from Roche. Oligonucleotides were obtained from the Nucleic Acids and Peptide Sequencing (NAPS) unit at UBC and repurified by 10–20% 8 M urea denaturing PAGE. Sequenase 2.0, pyrophosphatase, and Sephadex G-25 were obtained from Amersham. $Hg(OAc)_2$ and other buffer salts were obtained from Aldrich.

Synthetically modified, self-cleaving DNA was prepared enzymatically by primer extension as previously described.[47] Briefly, 20 pMol of primer containing an embedded ribose, rC, (5′-biotin-T_{20}GCGTGCC**r**CGTCTGTTGGGCCC-3′) was annealed to template (5′-T_9GAGCTCGCGGGGCGTGCC-

TTCACTACGGATGAGAACTGTTGGTAGGGCCCA ACAGAGGGCACGCTCGTGTCGT-3′), then enzymatically polymerized at 37 °C using Sequenase 2.0 in the presence of pyrophosphatase, 50 μM $dA^{im}TP$, $dU^{aa}TP$, dCTP, dGTP, and trace amounts of dGTP α-[^{32}P]. The reaction was stopped by addition of EDTA (25mM final), then desalted on a G-25 spin column for use in kinetic studies.

For first-order kinetic analysis of self-cleavage, trace amounts (~500 kcpm, or an estimated 2–5 pmol) of the self-cleaving DNA were bound (via 5′-biotin) to prewashed avidin magnetic particles. The template strand was then removed by five short washes of 100 μL 0.2 M NaOH, followed by a neutralization wash of 100 μL 100 mM cacodylate (pH 6.5) and then 100 μL water wash. When suspended in water or 10 mM buffer in the absence of NaCl, a slurry could be stored for up to 2 hours without significant cleavage being observed. The slurry of avidin particles in water were then divided into several tubes, decanted, then resuspended and incubated for 30 min in 100 μL of a solution containing metal cation of interest at various final cleavage reaction ("1×") concentrations. Following this preincubation, the avidin particles were separated by magnetization. The beads were again resuspended in 1× metal cation solution, whereupon cleavage reactions were initiated by addition of 5 μL of slurry to 5 μL of 2× cleavage buffer (400 mM NaCl, 50 mM cacodylate pH 7.5) also containing 1× metal cation. The experiment was also repeated without the 30 min preincubation with metal cation, with no significant difference in cleavage rates. Kinetics were performed at room temperature that was recorded at 23 °C.

For rescue experiments, 1 μL of either 0.1 M DTT or 0.5 M EDTA was added to the reaction at time zero. Reactions were incubated at 23 °C, then quenched at indicated times by addition of 15 μL formamide (containing 20 mM EDTA, 4 mM biotin, 0.01% bromophenol blue, 0.01% xylene cyanole). Samples were heated to 95 °C for 5 min, then immediately resolved by 10% 8 M urea denaturing PAGE. Product formation was visualized using a phosphorimager (Amersham Typhoon 9200), and quantified using the ImageQuant 5.2 program.

For the initial survey of metal cations, just one time point was taken at 10 min for three different concentrations of each metal cation (10, 30, and 100 μM); a control reaction (absent any inhibitory metal cations) with eight time points was also conducted at the same time to verify that 9_{25}–11 was fully active. Mercury and copper inhibition was further characterized by measuring initial rates over the first 15 min of the cleavage reaction at various Hg^{2+} and Cu^{2+} concentrations. To verify that the inhibition was reversible and that the metal cation binding was in rapid equilibrium, extent of cleavage was also monitored in the presence of Hg^{2+} by comparing the fractions cleaved at the endpoints of the control reaction and a reaction carried out in the presence of 3 μM Hg^{2+} for which time points were taken over 50 h.

Hill-plot analysis was performed as previously described for a self-cleaving ribozyme.[48] First order rate constants (k_{obs}) were determined by fitting the fraction cleaved to Eqn. 1 using Sigma Plot 2001 (version 7.101) based on a steady state assumption for accumulation of active self-cleaving species.

$$[P]_t = [P]_\infty (1 - e^{-k_{obs}t}) \quad (1)$$

where $[P]_t$ and $[P]_\infty$ are the fractions cleaved at time t and the end point respectively. As it was determined the Hg^{2+} inhibited reactions eventually proceed to completion, $[P]_\infty$ was fixed to the value determined for the control reaction for the Hg^{2+} inhibited reactions. The apparent dissociation constant (K_d^{APP}) for Hg^{2+}, were then determined from the slope and intercept, respectively, obtained from fitting a plot of k_{obs}^{-1} *vs.* $[Hg^{2+}]$ to eqn. 2:

$$\frac{1}{k_{obs}} = \frac{k_{off} + k_o}{k_{off} k_o} + \frac{[Hg^{2+}]}{k_o K_d^{APP}} \quad (2)$$

where k_o is fixed to the value of observed rate constant for the control reaction in the absence of mercury. Eqn. 2 is derived from the kinetic scheme and derivation shown below:

$$DNA_{cleaved} \xleftarrow{k_o} DNA_{free} + Hg^{2+} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} DNA_{bound} \bullet Hg^{2+}$$

Assuming a steady state for DNA_{free} in the above kinetic scheme, eqn. 3 is obtained:

$$k_{on}[DNA_{free}][Hg^{2+}] + k_o[DNA_{free}] = k_{off}[DNA_{bound} \cdot Hg^{2+}] \quad (3)$$

Combining eqn. 3 with the mass balance eqn. 4, where [U] is the uncleaved DNA that can be measured at any given time, yields eqn 5:

$$[U] = [DNA_{free}] + [DNA_{bound} \cdot Hg^{2+}] \quad (4)$$

$$[U] = [DNA_{free}]\left(1 + \frac{k_{on}[Hg^{2+}] + k_o}{k_{off}}\right) \quad (5)$$

where [U] denotes total uncleaved DNA. Solving eqn. 5 for $[DNA_{free}]$ and inserting this result into the first order rate equation yields eqn. 6:

$$\frac{dP}{dt} = -\frac{d[DNA_{free}]}{dt} = k_o[DNA_{free}] = \left(\frac{k_o k_{off}}{k_{off} + k_{on}[Hg^{2+}] + k_o}\right)[U] = k_{obs}[U] \quad (6)$$

where k_{obs}^{-1} corresponds to eqn. 2.

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