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Toward an RNaseA Mimic: A DNAzyme with Imidazoles and Cationic Amines

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RNA cleavage has received considerable attention over the years. Early biomimetic studies looked to protein nucleases for inspiration in the rational design of small molecules that chelated metals, or positioned imidazoles and other functionalities to mimic the active site of RNaseA.[1] These studies were extended to synthetic oligonucleotides that hydrolyze complementary RNA molecules.[2] Oligonucleotides appended with metal chelators cleave RNA relatively efficiently.[3] It has been far more challenging to append imidazoles and cationic amines to cleave RNA in the absence of divalent metals. Many reports underscore the importance of imidazole/amine-appended oligonucleotides to medicine and biology. Few describe multiple catalytic turnover.[4]

Whereas directed synthesis continues in rationally tackling the challenge of ribophosphodiester cleavage, combinatorial nucleic acid selection, which allows for massive parallel sampling of potential catalysts, has led to the discovery of new ribozymes and DNAzymes.[5,6] Almost without exception,[7] combinatorially selected RNA-cleaving ribozymes and DNAzymes depend on divalent metal cations (M^{2+}). Two combinatorially selected, M^{2+}-independent "DNAzymes" (40−50nt motifs) that self-cleave in 0.25−1 M monovalent cations at pH 7, displayed rate constants on the order of 10^{-3} to 10^{-4} min^{-1}.[8] In both cases, neither *in trans* catalysis nor multiple turnover was observed in the absence of a M^{2+}. Together these important studies identified the "intrinsic" catalytic nature of DNA. Similar results were found with the 86nt HDV ribozyme that normally operates with Mg^{2+}; in the absence of a M^{2+}, the k_{cat} at pH 7 for HDV ribozyme self-cleavage fell below 1×10^{-3} min^{-1} and the pH-rate optimum fell below 7.[9] The significant diminution of rate constants **at pH > 7 in the absence of a M^{2+}** is most likely due to the fact that nucleic acids lack a cationic functionality and ineffectively perturb extant nucleobase pK_a's requisite for general acid/base catalysis.[10] This has been explained by the lack of functional groups endemic to proteins and the roles they play in both catalysis and folding.[11]

Numerous reports have recently underscored the potential of synthetically appending dNTPs with imidazoles, cationic amines, or other functionalities for use in a combinatorial selection.[12] There have been far fewer reports of true catalytic activity based on combinatorially selected *modified* RNA and DNA. A copper-dependent, pyridyl-modified RNA Diels−Alderase was the first modified nucleic acid catalyst.[13] Two notable examples with appended imidazoles are (1) a Zn-dependent RNase[14] and (2) a Cu-dependent amide synthase.[15] A ligase−RNA appended with amines has also been reported.[16] All of these are M^{2+}-dependent. There has been only one report to date where *both* cationic amines *and* imidazoles necessarily act in concert to hydrolyze an intramolecular ribophosphodiester linkage, and this activity proceeded in the *absence* of M^{2+}.[17] Nevertheless, no evidence was presented for *trans* cleavage, specificity, and turnover. Here we report the first truly catalytic, M^{2+}-independent DNAzyme with two protein-like functionalities that cleaves RNA with both turnover and high specificity. This motif, 9_{25}-11, derived from the combinatorially selected self-cleaving sequence, (Figure 1) is one of the smallest M^{2+}-independent catalysts reported to date.

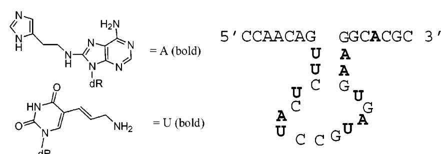

Figure 1.

This work now demonstrates how synthetic organic chemistry, when merged with combinatorial selection, can result in a new class of DNAzymes that meets the ongoing synthetic challenges for developing relatively small biomimetic catalysts. Having met this challenge, 9_{25}-11 is a unique template for structure−activity studies to characterize general-acid/base RNA hydrolysis without entertaining the complicating effects of divalent metals.[18]

9_{25}-11 was synthesized by standard solid-phase protocols using phosphoramidite precursors synthesized according to precedent (see Supporting Information).[19] Three other sequences were also synthesized to better characterize the catalytic motif (Table 1): (**1**) the full-length catalyst 9_{25}-11, (**2**) a hypothetical "minimal" motif composed of just two proximal histaminyl-dA's and two proximal amino-allyl-dU's, (**3**) a deletion construct lacking three bases at positions 14−16, (**4**) the full-length unmodified sequence.

Table 1. Sequences Listed 5′ to 3′ [a]

1:	CCAACAG**UU**C**U**C**AU**CCG**UA**G**U**G**AA**GGC**A**CGC
2:	CCAACAG**UU**CCUUCGGG**AA**GGCACGC
3:	CCAACAG**UU**C**U**C**AU**CCG**UA**G**A**GGC**A**CGC
4:	CCAACAGTTCTCATCCGTAGTGAAGGCACGC

[a] Bold signifies modified.

Only the full-length 9_{25}-11 (**1**) supported catalysis using the 5′ ^{32}P-d(GCGTGCC)rCd(GTCTGTT) substrate (Figure 2 and Supporting Information). The active site appears to be more complex than simply two U's and two A's anchored in proximity to the target ribose by a "GC staple". Although the U and A at positions 14 and 16 also appear to be essential to catalysis as the deletion construct was inactive, they may be simply required for folding.

Single-turnover kinetics (Figure 2, lanes 1−5) with **1** resulted in >80% cleavage *in trans* after 5 h. A k_{cat} of $\sim 1.5 \times 10^{-2}$ min^{-1} was calculated from a first-order rate expression (assuming $[E_T]k_{on} \gg k_{cat}$). This is diminished 3-fold from that observed previously for intramolecular cleavage. An identical substrate that was "degenerate" for ribose (rN signifies equal representation of all four ribonucleosides) provided a measure of the specificity for target ribonucleoside recognition.[20] The catalyst cleaved only 25% of the degenerate substrate, indicating high substrate specificity (Figure 2, lanes 8−12).[21] We also observed 13−14 turnovers at 2.5 μM substrate and 100 nM catalyst (Figure 2, lanes 15−19). A

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k_{cat} of $\sim 9 \times 10^{-3} \pm 7 \times 10^{-4}$ min^{-1} ($k_{cat} = V/E_T$ taken at 3, 5, 10, 24 h) generally agrees with the value from the single-turnover experiment.[22] Since no exogenously added cofactor was necessary to initiate cleavage, we experimentally excluded the possibility that a small amount RNaseA could have contaminated the purification of **1** (data in Supporting Information).[23]

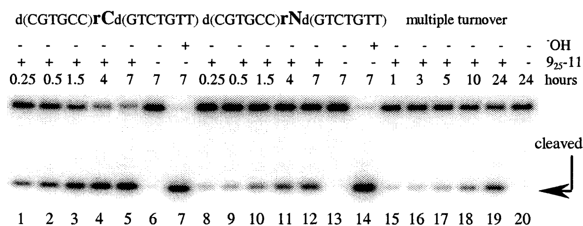

Figure 2. (Lanes 1−5) Single-turnover kinetics (2.5 μM catalyst, 0.25 μM substrate 30 °C) on specific substrate 0.25−7 h. (Lane 6) No catalyst, 7-h control. (Lane 7) NaOH treatment. (Lanes 8−12) Single-turnover kinetics on degenerate substrate at 0.25−7 h. (Lane 13) No catalyst, 7-h control. (Lane 14) NaOH treatment. (Lanes 15−19) Multiple-turnover kinetics (2.5 μM substrate, 100 nM catalyst 27 °C) 1−24 h. (Lane 20) No catalyst, 24-h control; 5-μL reactions run in 500 mM NaCl, 50 mM Tris-HCl pH 7.9, 1 mM EDTA, quenched with formamide and resolved by 20% PAGE, no heat.

Directed synthesis to append imidazoles or amines or both to oligonucleotides so as to target RNA cleavage represents an exciting avenue of research. Nevertheless, single-turnover cleavage, if observed at all, is often significantly slower than that observed for **1**, whereas multiple turnover, to the best of our knowledge, has never been reported irrespective of ionic strength. 9_{25}-11 is the first example of a M^{2+}-independent DNAzyme that is fully catalytic at pH 7.9. This report follows that of a M^{2+}-independent self-cleaving RNase activity ($k_{obs} \approx 0.045$ min^{-1}, 200 mM NaCl) that had initially been sought to gauge the catalytic boost that might be achieved by delivering synthetic functionality to combinatorial selections. This boost was found to be worth 1−3 orders of magnitude when rate constants were compared to those of significantly longer, naturally occurring, and combinatorially selected self-cleaving species at similar ionic strength. In the context of *trans* cleavage and turnover, comparison to natural ribozymes is more difficult as *trans* cleavage at pH 7 in the absence of a M^{2+} may only be observed at 1−4 M monovalent cations[24] or in the presence of various polyamines.[25] At 1 M monovalent cations, a His-dependent, 30nt DNAzyme acts similarly to 9_{25}-11.[26] The work herein suggests an improvement over the development of synthetic conjugates and a new approach to complement the study of unmodified catalysts. This motif, as the first of its kind, is small yet structurally and chemically unique. Systematic replacement of histaminyl-dA with imidazole isosteres along with chemical rescue will be required to fully determine the structural basis for catalysis.[27,28] Whether the imidazoles and amines are involved directly in catalysis rather than simply in folding and pK_a perturbation of C or G will be of paramount importance to determine if 9_{25}-11 is a true RNaseA mimic with a dedicated pair of catalytic imidazoles. Reselection from libraries of greater length and degeneracy or with differently modified dNTPs[29] may result in more efficient and more complex catalysts.

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Supporting Information Available: Experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Sigman, D. S.; Wahl, G. M.,; Creighton, D. J. *Biochemistry* **1972**, *11*, 2236. (b) Breslow, R.; Doherty, J. B.; Guillot, G.; Lipsey, C. *J. Am. Chem. Soc.* **1978**, *100*, 3227−3229. (c) Hirst, S. C.; Tecilla, P.; Geib, S. J.; Fan, E.; Hamilton, A. D. *Isr. J. Chem.* **1992**, *32*, 105.
(2) References reviewed in: (a) Trawick, B. N.; Daniher, A. T.; Bashkin, J. K. *Chem. Rev.* **1998**, *98*, 939−960. (b) Oivanen, M.; Kuusela, S.; Lönnberg, H. *Chem. Rev.* **1998**, *98*, 961.
(3) (a) Trawick, B. N.; Osiek, T. A.; Bashkin, J. K. *Bioconj. Chem.* **2001**, *12*, 900. (b) Hall, J.; Hüsken, D.; Häner, R. *Nucleic Acids Res.* **1996**, *24*, 3522, (c) Bashkin, J. K.; Frolova, E. I.; Sampath, U. *J. Am. Chem. Soc.* **1994**, *116*, 5981.
(4) (a) Komiyama, M.; Inokawa, T. *J. Biochem.* **1994**, *116*, 719. (b) Reynolds, M. A.; Beck, T. A.; Say, P. B.; Schwartz, D. A.; Dwyer, B. P.; Daily, W. J.; Vaghefi, M. M.; Metzler, M. D.; Klem, R. E.; Arnold, L. J. *Nucleic Acids Res.* **1996**, *24*, 760. (c) Ushijima, K.; Gouzu, H.; Hosono, K.; Shirakawa, M.; Kagosima, K.; Takai, K.; Takaku, H. *Biochim. Biophys. Acta* **1998** *1379*, 217. (d) Beloglazova, N. G.; Sil'nikov, V. N.; Zenkova, M. A.; Vlassov, V. V. *FEBS Lett*. **2000**, *481*, 277. (e) Verbeure, B.; Lacey, C. J.; Froeyen, M.; Rozenski, J.; Herdewijn, P. *Bioconj. Chem.* **2002**, *13*, 333.
(5) (a) Ellington, A. D.; Szostak, J. W. *Nature* **1990**, *346*, 818. (b) Tuerk, C.; Gold, L. *Science* **1990**, *249*, 505. (c) Beaudry, A. A.; Joyce, G. F.; *Science* **1992**, *257*, 635.
(6) Breaker, R. R.; Joyce, G. F. *Chem. Biol.* **1994**, *1*, 223.
(7) Exceptionally, there is a His-dependent DNAzyme: Roth, A.; Breaker, R. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 6027.
(8) (a) Geyer, C. R.; Sen, D. *Chem. Biol.* **1997**, *4*, 579. (b) Faulhammer, D.; Famulok, M. *J. Mol. Biol.* **1997**, *268*, 188.
(9) (a) Wadkins, T. S.; Shih, I.-h.; Perrotta, A. T.; Been, M. D. *J. Mol. Biol.* **2001**, *305*, 1045. (b) Perrotta, A. T.; Shih, I. H.; Been, M. D. *Science* **1999**, *286*, 123.
(10) Jayasena, V. K.; Gold, L. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10612.
(11) Narlikar, G. J.; Herschlag, D. *Annu. Rev. Biochem.* **1997**, *66*, 19.
(12) (a) Benner, S. A. et al. *Pure Appl. Chem.* **1998**, *70*, 263. (b) Sakthivel, K.; Barbas, C. F. *Angew. Chem., Int. Ed.* **1998**, *37*, 2872. (c) Perrin, D. M.; Garestier, T.; Helene, C. *Nucleosides Nucleotides* **1999**, *18*, 377. (d) Matulic-Adamic, J.; Daniher, A. T.; Karpeisky, A.; Haeberli, P.; Sweedler, D.; Beigelman, L. *Bioorg. Med. Chem. Lett*. **2000**, *10*, 1299. (e) Vaish, N. K.; Fraley, A. W.; Szostak, J. W.; McLaughlin, L. W. *Nucleic Acids Res.* **2001**, *28*, 3316. (f) Sawai, H.; Ozaki, A. N.; Satoh, F.; Ohbayashi, T.; Masud, M. M.; Ozaki, H. *Chem. Commun.* **2001**, *24*, 2604. (g) Gourlain, T.; Sidorov, A.; Mignet, N.; Thorpe, S. J.; Lee, S. E.; Grasby, J. A.; Williams, D. M. *Nucleic Acids. Res.* **2001**, *29*, 1898. (h) Thum, O.; Jäger, S.; Famulok, M. *Angew. Chem., Int. Ed.* **2001**, *40*, 3990.
(13) Tarasow, T. M.; Tarasow, S. L.; Eaton, B. *Nature* **1997**, *389*, 54.
(14) Santoro, S. W.; Joyce, G. F.; Sakthivel, K.; Gramatikova, S.; Barbas, C. F. *J. Am. Chem. Soc.* **2000**, *122*, 2433.
(15) Wiegand, T. W.; Janssen, R. C.; Eaton B. *Chem. Biol.* **1997**, *4*, 675.
(16) Teramoto, N.; Imanishi, Y.; Ito, Y. *Bioconj. Chem.* **2000**, *11*, 744.
(17) Perrin, D. M.; Garestier, T.; Helene, C. *J. Am. Chem. Soc.* **2001**, *123*, 1556.
(18) Nakano, S.; Bevilacqua, P. C. *J. Am. Chem. Soc*. **2001**, *123*, 11333.
(19) (a) Cook, A. F.; Vuocolo, E.; Brakel, C. L. *Nucleic Acids Res.* **1988**, *16*, 4077. (b) Bashkin, J. K.; McBeath, R. J.; Modak, A. S.; Sample, K. R.; Wise, W. B. *J. Org. Chem.* **1991**, *56*, 3168. (c) Bashkin, J. K.; Sondhi, S. M.; Sampath, U. S.; d'Avignon, D. A.; Modak, A. S. *New J. Chem.* **1994**, *18*, 305. (d) Lermer, L.; Hobbs, J.; Perrin, D. M. *Nucl. Nucl. Nucl. Acids* **2002**. In press.
(20) Santoro, S.; Joyce, G. F. *Biochemistry* **1998**, *37*, 13330.
(21) At lower temperatures (27 °C), selectivity fell 2-fold, whereas at higher temperatures (33−36 °C) the selectivity remained the same but product formation slowed as the substrate's T_m is estimated to be 32−35 °C, and kinetics were no longer likely to be first-order in nature (T_m = 40 °C in 0.9 M SSC, no penalty assessed for unpaired dG, and dT).
(22) Calculated at 1 h, a k_{cat} of 1.9×10^{-2} min^{-1} is in better agreement with other values obtained from the single-turnover experiment.
(23) Although RNase contamination was unlikely since the degenerate substrate was cleaved ~25%, we repeated kinetics with a 5′-labeled 75nt all-RNA sequence complementary to the substrate to show that the PAGE-purified catalyst neither displayed a nonspecific RNase activity nor contained a contaminating one (see Supporting Information).
(24) (a) Curtis, E. A.; Bartel, D. P. *RNA* **2001**, *7*, 546. (b) O'Rear, J. L.; Wang, S.; Feig, A. L.; Beigelman, L.; Uhlenbeck, O. C.; Herschlag, D. *RNA* **2001**, *7*, 537. (c) Murray, J. B.; Seyhan, A. A.; Ng, W.; Burke, J. M.; Scott, W. G. *Chem. Biol.* **1998**, *5*, 587.
(25) (a) Chowrira, B. M.; Berzal-Herranz, A.; Burke, J. M. *Biochemistry* **1993**, *32*, 2, 1088. (b) Earnshaw, D. J.; Gait, M. J. *Nucleic Acids Res.* **1996**, *26*, 5551.
(26) Roth, A.; Breaker, R. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 6027.
(27) (a) Tarasow, T. M.; Tarasow, S. L.; Tu, C.; Kellogg, E.; Eaton, B. E. *J. Am. Chem. Soc.* **1999**, *121*, 3614. (b) Oyelere, A. K.; Kardon, J. R.; Strobel S. A. *Biochemistry* **2002**, *41*, 3667.
(28) (a) Peracchi, A.; Beigelman, L.; Usman, N.; Herschlag, D. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 11522. (b) Perrotta, A. T.; Shih, I. H.; Been, M. D. *Science* **1999**, *286*, 123.
(29) Gourlain, T.; Sidorov, A.; Mignet, N.; Thorpe, S. J.; Lee, S. E.; Grasby, J. A.; Williams, D. M. *Nucleic Acids Res.* **2001**, *29*, 1898.

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