Merely two mutations switch a DNA-hydrolyzing deoxyribozyme from heterobimetallic (Zn^{2+}/Mn^{2+}) to monometallic (Zn^{2+}-only) behavior†

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A deoxyribozyme that hydrolyzes DNA phosphodiester linkages with a requirement for both Zn^{2+} and Mn^{2+} is switched by only two nucleotide mutations to require Zn^{2+} alone, demonstrating that DNA-catalyzed DNA hydrolysis can be achieved using only one metal ion cofactor.

Metal ions can significantly expand the scope of macromolecular catalysis. For catalysts composed of nucleic acids (DNA or RNA), metal ions are typically required for both structural and catalytic roles. Divalent metal ions such as Mg^{2+}, Mn^{2+}, and Zn^{2+} are often, but not always,[1–3] obligatory cofactors for ribozymes and deoxyribozymes. We recently described the 10MD5 deoxyribozyme as an example of a highly active DNA catalyst that hydrolyzes DNA phosphodiester linkages of single-stranded DNA substrates.[4] Because uncatalyzed P–O bond hydrolysis is extremely slow ($t_{1/2} \approx 30$ million years),[5] the hydrolysis achieved by 10MD5 ($t_{1/2} \approx 15$ min) represents a considerable 10^{12} rate enhancement. 10MD5 was identified by *in vitro* selection[6] in the presence of a mixture of Mg^{2+}, Mn^{2+}, and Zn^{2+}; its activity requires both Mn^{2+} and Zn^{2+}. The $K_{d,app}$ for Mn^{2+} is $\sim$5 mM and the optimal Zn^{2+} concentration is 1.0 mM, with significant loss of rate and yield at either lower or higher Zn^{2+} concentrations. Another salient characteristic of 10MD5 is its rather sharp pH optimum.[4] Catalysis is optimal near pH 7.5; pH changes of merely 0.2 units in either direction lead to a much lower DNA hydrolysis rate constant (at least six-fold decrease) and yield (at least two-fold decrease). We subsequently used *in vitro* evolution to identify a quintuple mutant of 10MD5, named 9NL27, that is more tolerant to a range of pH values.[7] In the present study, we evaluated the divalent metal ion requirements of 10MD5, 9NL27, and related sequence variants. We found that only two nucleotide mutations in 10MD5 are sufficient to switch the metal ion dependence from heterobimetallic (Zn^{2+} and Mn^{2+}) to strictly monometallic (Zn^{2+} only), with both practical and mechanistic implications.

The 9NL27 deoxyribozyme is one of several 10MD5 variants that emerged upon *in vitro* evolution of 10MD5 for improved pH tolerance.[7] 9NL27 and 10MD5 differ at only five out of 40 nucleotides in the originally random enzyme region, which is located between two fixed binding arm sequences (Fig. 1a). Whereas both 10MD5 and 9NL27 have comparable DNA hydrolysis activities in the presence of 1 mM Zn^{2+} and 20 mM Mn^{2+} (in 70 mM HEPES, pH 7.5, 150 mM Na^+ at 37 °C; Fig. 1b), the observed catalytic properties change under

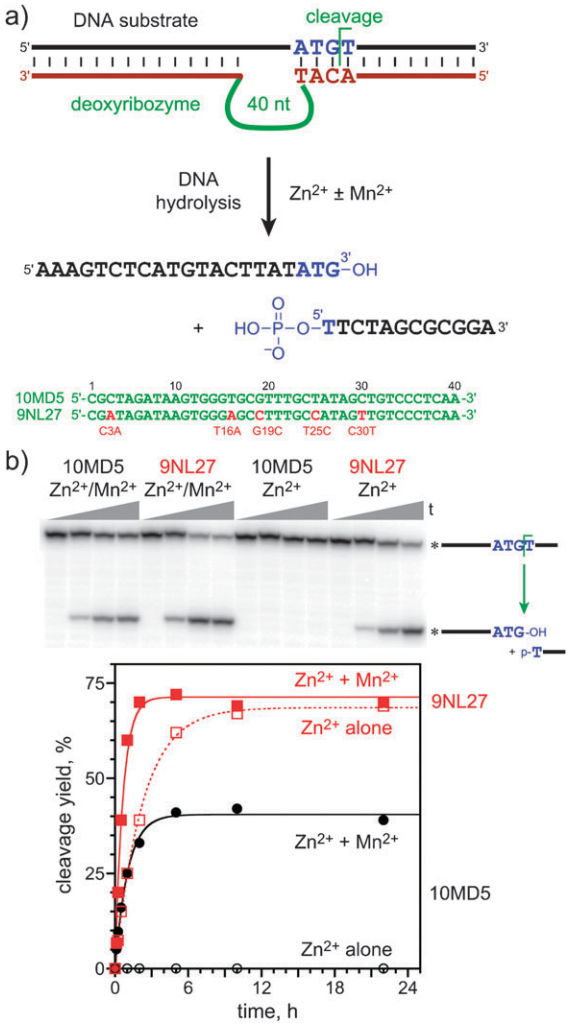

Fig. 1 The 10MD5 and 9NL27 deoxyribozymes that hydrolyze DNA. (a) Depiction of the complex between deoxyribozyme and DNA substrate. Sequences of the 40-nucleotide enzyme regions of 10MD5 and 9NL27; the latter has five mutations relative to the former. For 10MD5, the DNA substrate may have essentially any sequence outside of the ATG^T "recognition site", as long as Watson–Crick base pairing is maintained with the deoxyribozyme.[4] For 9NL27, the recognition site is TATG^TT.[7] (b) Assays of 10MD5 and 9NL27 with Zn^{2+} and Mn^{2+}, or with Zn^{2+} alone.‡ t = 0, 15 min, 2 h, 22 h for PAGE image. *Conditions*: 70 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM $ZnCl_2$ ± 20 mM $MnCl_2$ as indicated, 37 °C. k_{obs} values (h^{-1}, top to bottom): 1.56, 0.45, 0.96.

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other ionic conditions. In the presence of 1 mM Zn^{2+} alone, 10MD5 is essentially inactive (<0.5% DNA hydrolysis in 22 h; Fig. 1b). In contrast, 9NL27 still functions with only a 3-fold reduction in k_{obs} relative to activity with both Zn^{2+} and Mn^{2+} (Fig. 1b).

To understand these functional differences, here we investigated the contributions of the five nucleotides (C3, T16, G19, T25, and C30) that differ between 10MD5 and 9NL27. By examining two quadruple mutants in which either C3 or T25 remained unmutated relative to the 10MD5 parent sequence, the C3A and T25C mutations found in 9NL27 were shown to be dispensable for Zn^{2+}-only activity (assays 1 and 2 in Fig. 2). The remaining three mutations found in 9NL27, T16A, G19C, and C30T, enabled Zn^{2+}-only activity (assay 3). Then, the two T16A and G19C mutations were together shown to be sufficient for Zn^{2+}-only activity, with at most slight assistance from the C30T mutation. With T16A or G19C alone, no cleavage activity was observed (assays 4 and 5), whereas the T16A/G19C double mutant—now designated as 10MD5-AC for brevity—was substantially active (assay 6). In contrast, the C30T mutation was clearly not required for Zn^{2+}-only activity (assay 6). The G19C/C30T double mutant was entirely inactive while the T16A/C30T double mutant had only trace activity at long incubation times (only ~2% yield in 22 h; Fig. S1 in the ESI†), suggesting that C30T can substitute only very ineffectively for G19C. In a full kinetics experiment, 10MD5-AC was observed to have k_{obs} of 2.5 h^{-1} with Zn^{2+} and Mn^{2+} and 0.037 h^{-1} (*i.e.*, about 70-fold lower) with Zn^{2+} alone (data not shown).

Because 9NL27 was originally identified on the basis of its broad pH tolerance using Zn^{2+} and Mn^{2+},[7] we evaluated the pH tolerance of 9NL27 and 10MD5-AC, each with Zn^{2+} and Mn^{2+} or with Zn^{2+} alone. For both deoxyribozymes, although a combination of Zn^{2+} and Mn^{2+} supported broad pH tolerance, the tolerance was lower with Zn^{2+} alone (Fig. 3). Including 40 mM Mg^{2+} with the Zn^{2+} did not improve the pH tolerance (data not shown). Therefore, the single set of mutations T16A/G19C does not simultaneously enable Zn^{2+}-only activity and broad pH tolerance by the 10MD5 deoxyribozyme. Whether both of these features are possible at the same time for other catalytic DNA sequences must be determined in future experiments.

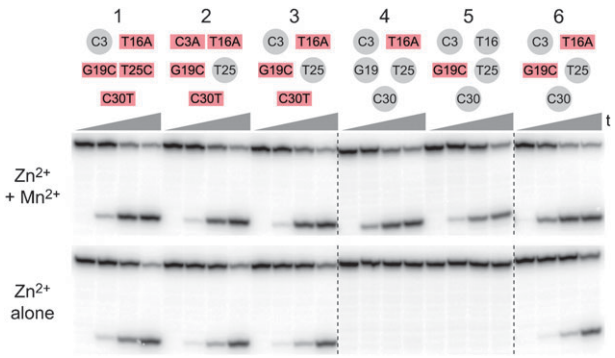

Fig. 2 DNA hydrolysis assays for 10MD5 mutants at pH 7.5 and 1 mM Zn^{2+} ± 20 mM Mn^{2+}. Conditions as in Fig. 1 with t = 0, 15 min, 2 h, 22 h. For each of assays 1–6, grey circles denote nucleotides unmutated from those found in the parent 10MD5 deoxyribozyme, and red rectangles denote nucleotides mutated in the 9NL27 deoxyribozyme. In assay 6, the double mutant (which has the two T16A and G19C mutations sufficient for activity with Zn^{2+} alone) is designated as 10MD5-AC.

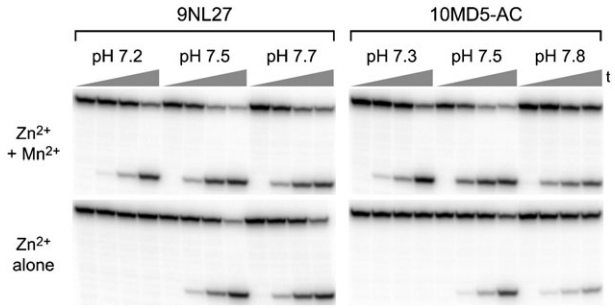

Fig. 3 Assaying the pH tolerance of 9NL27 and 10MD5-AC, each with 1 mM Zn^{2+} ± 20 mM Mn^{2+}. Conditions as in Fig. 1 with t = 0, 15 min, 2 h, 22 h. The pH optima of the two deoxyribozymes appear to be slightly different. Because we observe precipitation of Zn^{2+} above pH 7.8, we could not assay 9NL27 at higher pH values than illustrated to ascertain whether the pH optimum with Zn^{2+} alone has shifted substantially to higher values.

The activity of the parent 10MD5 deoxyribozyme is sharply dependent on Zn^{2+} concentration.[4] We examined the Zn^{2+} concentration dependence of the 10MD5-AC double mutant at pH 7.5, both in the presence and absence of Mn^{2+} (Fig. 4). 10MD5-AC was found to tolerate a substantially broader range of Zn^{2+} concentrations than the parent 10MD5 (although only in the presence of Mn^{2+}, similar to the situation with pH tolerance as shown in Fig. 3). Therefore, the sharp Zn^{2+} optimum of 10MD5 is not an inherent requirement of DNA-catalyzed DNA hydrolysis. A reselection experiment starting with the partially randomized 10MD5 sequence and selecting directly for tolerance of a wider range of Zn^{2+} concentrations led to numerous 9NL27-like sequences, uniformly including mutations at the key T16 and G19 positions (Fig. S2 in the ESI†). We also showed that the

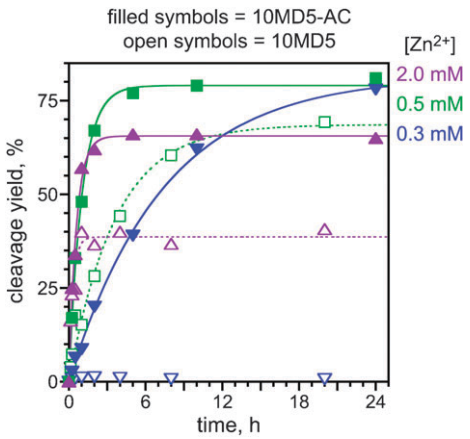

Fig. 4 The 10MD5-AC double mutant tolerates a broader range of Zn^{2+} concentrations than does the parent 10MD5 deoxyribozyme. Data were obtained at pH 7.5 and 20 mM Mn^{2+} with the indicated [Zn^{2+}]: 0.3 mM, ▼/▽; 0.5 mM, ■/□; 2.0 mM, ▲/△. Note that at each illustrated [Zn^{2+}], the DNA cleavage rate, yield, or both were higher for 10MD5-AC than for 10MD5. k_{obs} values (h^{-1}) were as follows. 10MD5-AC: 0.3 mM Zn^{2+}, 0.14; 0.5 mM Zn^{2+}, 0.98; 2.0 mM Zn^{2+}, 1.7. 10MD5: 0.5 mM Zn^{2+}, 0.28; 2.0 mM Zn^{2+}, 3.2.

10MD5-AC double mutant, like 9NL27 itself,[7] retains high cleavage site specificity when the recognition site is expanded by two nucleotides from ATGˆT (as found in the parent 10MD5 deoxyribozyme; Fig. 1) to TATGˆTT (Fig. S3 in the ESI†).

In summary, only two nucleotide mutations in the 10MD5 sequence, T16A and G19C, are required to convert the heterobimetallic (Zn^{2+}/Mn^{2+}) parent 10MD5 deoxyribozyme into a Mn^{2+}-independent deoxyribozyme that requires Zn^{2+} alone. This finding has three key implications. (1) In a practical context, the knowledge that robust DNA-catalyzed DNA hydrolysis can be achieved using only a single type of divalent metal ion cofactor (here, Zn^{2+}) suggests the pursuit of new monometallic DNA-hydrolyzing deoxyribozymes. Such efforts are underway. (2) In the context that heterobimetallic catalysis is a hallmark of certain natural protein nucleases,[8] our findings clarify that nucleic acid catalysts can facilitate even very difficult reactions such as DNA phosphodiester hydrolysis with the aid of just one type of divalent metal ion cofactor. (3) The new results have mechanistic implications for DNA hydrolysis by the 10MD5 family of deoxyribozymes. Plausibly, the catalytic mechanisms of all 10MD5-derived deoxyribozymes are similar. The present observations suggest that Mn^{2+} plays a noncatalytic structural role, considering its dispensable nature for catalysis by 9NL27 and 10MD5-AC, whereas Zn^{2+} likely plays a key catalytic role. Furthermore, nucleotides 16 and 19 of the enzyme region are likely to be key participants in metal ion binding during catalysis. High-resolution structural information is currently being sought to address these hypotheses.

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Notes and references

‡ DNA hydrolysis assays were performed as described previously.[4,7]

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