Substrate specificity and kinetic framework of a DNAzyme with an expanded chemical repertoire: a putative RNaseA mimic that catalyzes RNA hydrolysis independent of a divalent metal cation

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ABSTRACT

This work addresses the binding, cleavage and dissociation rates for the substrate and products of a synthetic RNaseA mimic that was combinatorially selected using chemically modified nucleoside triphosphates. This trans-cleaving DNAzyme, 925-11t, catalyzes sequence-specific ribophosphodiester hydrolysis in the total absence of a divalent metal cation, and in low ionic strength at pH 7.5 and in the presence of EDTA. It is the first such sequence capable of multiple turnover. 925-11t consists of 31 bases, 18 of which form a catalytic domain containing 4 imidazole and 6 allylamino modified nucleotides. This sequence cleaves the 15 nt long substrate, S1, at one embedded ribocytosine at the eighth position to give a 5' product terminating in a 2',3'-phosphodiester and a 3'-product terminating in a 5'-OH. Under single turnover conditions at 24°C, 925-11t displays a maximum first-order rate constant, $k_{\text{cat}}$, of 0.037 min$^{-1}$ and a catalytic efficiency, $k_{\text{cat}}/K_m$, of $5.3 \times 10^5$ M$^{-1}$ min$^{-1}$. The measured value of $k_{\text{cat}}$ under catalyst excess conditions agrees with the value of $k_{\text{cat}}$ observed for steady-state multiple turnover, implying that slow product release is not rate limiting with respect to multiple turnover. The substrate specificity of 925-11t was gauged in terms of $k_{\text{cat}}$ values for substrate sequence variants. Base substitutions on the scissile ribose and at the two bases immediately downstream decrease $k_{\text{cat}}$ values by a factor of 4 to 250, indicating that 925-11t displays significant sequence specificity despite the lack of an apparent Watson–Crick base-pairing scheme for recognition.

INTRODUCTION

The development of catalysts capable of sequence-specific RNA cleavage has received a great deal of attention over the past three decades (1,2). To date, there have been three general solutions to this goal: (i) synthetic conjugates, (ii) naturally occurring, self-cleaving ribozymes that have been engineered into true catalysts and (iii) combinatorially selected ribozymes and DNAzymes. These species may also be classified according to $M^{2+}$-dependence or $M^{2+}$-independence.

Although there are many examples of synthetically designed $M^{2+}$-dependent catalysts that are capable of catalytic turnover, the same cannot be said for synthetically designed $M^{2+}$-independent catalysts, which present a considerable challenge, particularly in terms of synthetic design where activity depends on the proper orientation of a combination of functionalities (e.g. imidazoles, guanidines or cationic amines). In the case of synthetic $M^{2+}$-independent catalysts, only a few examples exhibit sequence-specific cleavage at pH 7.5 at relatively low ionic strength (e.g. 100–200 mM $M^+$) (3), and none to date promotes measurable catalytic turnover.

In contrast to synthetic catalysts, naturally occurring ribozymes often manifest very high values for self-cleavage rate constants, particularly when $M^{2+}$ concentrations are high. However, these rate constants diminish when self-cleaving species are re-engineered for intermolecular cleavage (4). In cases where turnover has been investigated, the steady-state rate constant often drops even further as product release is often found to be rate limiting. Although the general requirement of a $M^{2+}$ for augmenting $k_{\text{cat}}$ in most ribozymes is well known, the question of $M^{2+}$-dependence versus $M^{2+}$-independence has been a source of debate. Initially, most ribozymes were considered to be absolutely $M^{2+}$-dependent, however $M^{2+}$-independence in some naturally occurring ribozymes has since been observed at high (1–4 M) monovalent ion concentration (5–7), with polyamines (8), or in the presence of the trivalent, exchange-inert cobalt hexamine (9–11), all of which suggest that the $M^{2+}$ is indirectly involved with structure and not necessarily with bond fission and formation (catalysis). In contrast to natural ribozymes, no study on $M^{2+}$-dependent DNAzymes has presented evidence to suggest that the $M^{2+}$ plays such an indirect role.

RNA-cleaving DNAzymes, which have recently garnered attention because of the enhanced nuclease stability of DNA, have been selected with diffusion-controlled catalytic efficiencies approaching $10^6$ M$^{-1}$ min$^{-1}$ when measured in the presence of liberal $M^{2+}$ concentrations (e.g. 10–100 mM Mg$^{2+}$, 10 mM Mn$^{2+}$, 3 mM Ca$^{2+}$, 10 $\mu$M Pb$^{2+}$ and 100 $\mu$M Zn$^{2+}$)
(12–15). In certain cases, $k_{cat}$ values may approach 10 min$^{-1}$ in the presence of moderate-to-high millimolar Mg$^{2+}$ or Mn$^{2+}$ concentrations (12,13,16). However, as the intracellular concentrations of the aforementioned free divalent cations are at least 2–3 orders of magnitude lower than those used to observe optimal catalytic activity, rate constants of 10 min$^{-1}$ cannot be expected under physiological conditions.

When DNA sequences have been found to be active in the absence of either a M$^{2+}$ or imidazole co-factor (17) for self-cleavage at an embedded ribose, $k_{cat}$ values are uniformly low (e.g. $k_{obs} \sim 10^{-3}–10^{-4}$ min$^{-1}$), and turnover has been observed in only one case (two turnovers in 100 h) (18–20). Similar rate constants and the absence of turnover were observed for the naturally occurring HDV ribozyme when examined in the absence of a M$^{2+}$, underscoring the overall importance (direct and indirect) of a M$^{2+}$ for efficient catalysis and turnover (21).

We have been interested in interfacing synthetic chemistry with combinatorial in vitro selection to address the alleged chemical dysfunction of catalytic nucleic acids that has been forwarded as an explanation for the generally unimpressive $k_{cat}$ values displayed by both natural ribozymes and in vitro selected ribozymes and DNAzymes (22). To this end, we took a cue from protein catalysts that marshal a wide range of chemical functionalities and often show catalytic rates that are several orders of magnitude superior to those of known catalytic nucleic acids. Of the 20 amino acid side chain functionalities, the two that are perhaps most conspicuously absent from the nucleic acid repertoire and yet quite commonly found at the active sites of protein catalysts that recognize phosphorylated substrates are the alkylammonium (Lys, $pK_a \sim 9.8$) and the imidazolium (His, $pK_a \sim 7.4$) ions. Quite a few studies have elaborated methodology for introducing these two functionalities, as well as several others, for use in combinatorial selections (23–37).

We hypothesized that these two functionalities would not only introduce protein-like functionality, but when properly oriented would impart a noteworthy catalytic advantage (35,38,39) that could be gauged against a stringent set of conditions (i–vii below) that has not to date been entirely satisfied by a catalyst of any genre. Using such an approach, we have been especially interested in developing a DNA-derived catalyst that uses added synthetic functionality to promote (i) sequence-specific ribophosphodiester hydrolysis, (ii) with turnover, (iii) at pH $\sim 7$, (iv) under relatively low monovalent ionic strength (e.g. 200 mM), (v) in the total absence of either a M$^{2+}$ or a polyamine, (vi) where $k_{cat}$ values surpass those of unmodified M$^{2+}$-independent DNAzymes when measured under any conditions and (vii) would compare favorably with other M$^{2+}$-dependent systems provided that the M$^{2+}$ is present at physiologically relevant levels (e.g. 0.5–2 mM Mg$^{2+}$) for the investigation of turnover, as opposed to self-cleavage. The reason for performing this was 2-fold: (i) initially the goal was to evaluate the extent of catalytic enhancement that might be observed with modified nucleotides under conditions where DNA is generally a poor catalyst and (ii) in so far as multiple catalytic turnover is a desirable quality for therapeutic anti-mRNA agents (e.g. ribozymes, DNAzymes and RNaseH-activated oligonucleotides), catalytic cleavage of RNA operating independently of a M$^{2+}$ might signify an advance for intracellular use where the availability of such cations may limit the activity of catalytic nucleic acids.

The result of this undertaking has been the independent and contemporaneous discovery of not one but two M$^{2+}$-independent, self-cleaving, DNA-derived, putative mimics of the protein RNaseA (40,41). The construct 925-11c is a M$^{2+}$-independent cis-cleaving DNA sequence (Figure 1A) that was selected using the monomer DNA polymerase substrates (Figure 1C), thus introducing imidazoles and amines into its catalytic core. This construct was converted to the trans-acting species, 925-11t (Figure 1B), by standard solid-phase DNA synthesis (42), endowing this species with the capacity for multiple turnover.

The work herein details the kinetics associated with 925-11t at 24°C in 50 mM Tris–HCl, pH 7.5, 1 mM EDTA and 200 mM NaCl. The target substrate, S1, is a 15 nt long oligonucleotide consisting of deoxyribonucleotides at all positions, except for a single embedded ribocytosine at the eighth base. 925-11t cleaves at the eighth base position of S1 to generate products with a 2’,3’-cyclic phosphate and a 5’-OH terminus. At 24°C, 925-11t displays a rate that is highly dependent on its target sequence and does not display a detectable rate for ligation.

**MATERIALS AND METHODS**

**Enzymes and chemicals**

Enzymes, $^{32}$P-labeled nucleotides, reagents and buffers were purchased from commercial sources. The 5-aminomethyldeoxyuridine and 8-histaminyl-deoxyadenosine phosphoramidites were synthesized as described previously (43).

**Oligonucleotides**

Substrate S1, 5’-GGTGCCrCCTCTGTTT-3’; non-cleavable substrate analogs S1-DNA and S1-OMe containing a cytosine deoxyribonucleotide and a 2’,3’-O-methylcytosine ribonucleotide respectively in place of the ribocytosine of S1; the substrate sequence variants listed in Table 1; precursor to the 5’-cleavage product of S1, pre-P$_S$, 5’-GGTGCCrCp-3’.

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![Figure 1](https://example.com/figure1.png)

**Figure 1.** (A) Upper left, Watson–Crick paired structures of the cis-cleaving species 925-11c. (B) Lower left, the trans-cleaving catalyst 925-11t and its target sequence S1. Bold type indicates modified DNA. Cleavage sites are indicated with arrows. (C) Right, Structures of the modified nucleotides: histaminyl-deoxyadenosine (A) or aminoallyl-deoxyuridine (U) used in the selection of 925-11c.
(where p is a 3'-phosphate); 3'-cleavage product of S1, P$_y$, 5'-GTCTGTTT-3' and the catalyst, 9$_y$-111, 5'-CCAACAGA-
GUUCCUAUUCCGUAUGAAAGGCACGC-3' (where U = 5-aminoallyl-deoxyuridine and A = 8-histaminy1-deoxyadeno-
sine). All oligonucleotides were synthesized using standard automated solid-phase methods on Applied Biosystems DNA synthesizers by the Nucleic Acids and Protein Synthesis unit at UBC and the University Core DNA and Peptide Services Unit at the University of Calgary. All labeled and unlabeled oligonucleotides were purified by electrophoresis using denaturing 20% (29:1, monomer:bis) polyacrylamide/ 7 M urea (D-PAGE) gels, ethanol precipitated, desalted on a G-25 spin column and stored in water prior to use.

The 5'-cleavage product of S1 possessing the 3'- (2',3'- cyclic phosphate), Pr, was synthetically prepared by incubating 30 nmol of Pre-P$_r$, in a 200 µl solution of 250 mM 2-((N-Morpholino) ethane sulfonic acid at pH 5.5 and 50 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride for 2 h at 37°C (44,45). Formation of the 2',3'-cyclic phosphate was verified by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF): [M-H]$^-$ m/z (predicted) = 2462.4, m/z (experimental) = 2460.6 (see Supplementary Material).

32P-oligonucleotide labeling

Substrate S1, its non-hydrolyzable analogs, substrates in Table 1 and the 5' cleavage product (P$_r$) were 5' end labeled with 32P using T4 polynucleotide kinase lacking 3'-phosphatase activity (Fermentas) and [γ-32P]ATP. 3',32P-labeled P$_y$ was prepared by annealing the oligo sequence 5'-GGCTGGCCr-CGCTCTG-3' with the complementary template 5'-T$_5$CAA-
CACGGGGCCACGC-3' and extending the duplex with Sequenase 2.0 (Amersham) and [α-32P]TTP. The product was confirmed by comparing its electrophoretic mobility with that of S1 in D-PAGE gels. The sample was subsequently treated with RNase A, and gel purified to give 3',32P-labeled P$_y$.

Kinetic experiments

The procedures used in the kinetic characterization of 9$_y$-11c and 9$_y$-11t were adapted from the procedures used in previous studies of catalytic RNAs (46-48). All experiments were performed under mineral oil to prevent non-specific binding and evaporation. Temperature varied by <0.5°C in a Julabo F-10 temperature controlled water bath. Reaction solutions and chases were buffered in CB200 (50 mM Tris–HCl, pH 7.5, 1 mM EDTA and 200 mM NaCl), except when 1 mM Mg$_{2+}$ was present in which case EDTA was absent. Solutions were preincubated at 24°C (or another temperature if specified) for a minimum of 1 h in order to ensure the reaction of only thermodynamically favored oligonucleotide folds. At least three independent data sets were collected for all experiments unless specified otherwise. Visualization of 32P-labeled complexes, substrates and products was accomplished by exposing gels on Molecular Dynamics phosphor screens at −20°C overnight and scanning the screens on a Molecular Dynamics Typhoon 9200 phosphoImager. Poly-
gons were drawn around distinct bands with ImageQuant v5.2. Quantified volumes were collected for background phosphorescence by ImageQuant v5.2 histogram peak correction prior to any mathematical treatment. The kinetic constants reported in this text are the result of non-linear least square fits of the cumulative plot of all data sets for identical experiments using the Sigma Plot 2001 v7.101 data analysis program. Errors associated with the constants reported in this paper are the standard errors generated from fits.

Pre-steady-state and single turnover experiments ($k_{obs}$)

For cleavage reactions, catalyst (at 2x concentration) and trace amounts of 32P-labeled substrate (<1 nM) were prepared separately in cleavage buffer at 24°C. Reactions were initiated by mixing equal volumes of substrate and catalyst solutions. Aliquots were taken at various time points, quenched with 2 vol of a 9:1 formamide:water, 50 mM EDTA, 0.01% bromophenol blue and 0.01% xylene cyanol loading solution and resolved by 20% D-PAGE. First-order rate constants, $k_{obs}$, were obtained by fitting data to the single exponential Equation 1: $[P]^t = [P]_0 \left(1 - e^{-k_{obs}t}\right)$

where $[P]^t$ and $[P]_0$ are the fractions of substrate cleaved at time $t$ and the end point, respectively.

Pulse-chase experiments to determine dissociation rate constants of S1 on denaturing gels ($k_{-1}$)

The 'pulse' phase was initiated by combining 1000 nM of catalyst (a saturating concentration of catalyst) with trace amounts (<1 nM) of 32P-labeled substrate at 24°C. The 'chase' phase of the reaction was initiated by the addition of a large excess of unlabeled S1 in CB200 to the pulse solution to give a mixture that is 100 µM in S1. Aliquots were quenched and resolved by D-PAGE as described above. The times between the pulse and the addition of the chase varied from 2 to 3 min. To correct for substrate cleavage that occurred between the pulse and chase times, a single turnover experiment was run in parallel at a saturating concentration of catalyst. Data from this control were fit to Equation 1 to obtain values for [P]$_0$ and $k_{cat STR}$ (single turn-
over). The fraction of S1 cleaved at the end of the pulse phase (values of [P], where $t$ varied from 2 to 3 min were interpolated from Equation 1 using the values of [P]$_0$ and $k_{cat STR}$ determined in the control reaction) was subtracted from the post-
chase data before it was fit to the single exponential Equation 1 to generate a first-order decay constant, $k_{chase}$ (4).

Multiple turnover experiments ($k_{cat MTR}$)

Steady-state experiments were carried out in multiple turnover reactions with excess S1 to catalyst ([S]/[E]>30) under ‘standard conditions’. Substrate was prepared by mixing trace amounts of 5',32P-labeled S1 with unlabeled S1. Catalyst preparation, preincubation of catalysts and substrates, reaction initiation, aliquot quenching and resolution by D-PAGE is as described for single turnover experiments. Multiple turn-
over kinetic data were fit to the equation:

$$P = k_{obs MTR}t$$

where $P$ was the concentration of S1 (in µM) cleaved at time $t$ (min) and $k_{obs MTR}$ is the observed steady-state rate of product formation (µM min$^{-1}$). At saturating concentrations of S1, values of $k_{obs MTR}$ obtained at different enzyme concentrations
were plotted versus catalyst concentration, [E]_T. Linear regression yielded a slope that represents the maximum first-order rate at saturating substrate concentrations, k_{cat\ MTR}, according to the following equation:

\[ k_{obs\ MTR} = \frac{[E]}{k_{cat\ MTR}} \]

Native gel-shift assays used for determining the dissociation rate constant of a substrate analog (k_{-1\ S1-OMe})

An aliquot of 1000 nM catalyst and a trace amount of 5'-32P-labeled S1-OMe were equilibrated together at 24°C for a minimum of 1.5 h. The experiment was initiated by the addition of an equal volume of a chase mixture, consisting of 100 μM unlabeled substrate in CB200. Aliquots were removed at different times and added to 1/10 vol of 70% sucrose, 0.01% bromophenol blue and 0.01% xylene cyanol in CB200 held in an ice bath. Time points were run 1–2 cm into a native 20% D-PAGE gel. The electrophoresis apparatus, gel and buffers were pre-cooled to 4°C to slow substrate dissociation that may occur as the complex runs through the gel. Owing to the high salt concentration, the electrophoresis buffer had to be recirculated in order to prevent detrimental pH changes.

The first-order dissociation rate constant, k_{-1\ S1-OMe}, was obtained by fitting data to the single exponential equation:

\[ f = f_0 + f_w \left(1 - e^{-k_{-1\ S1-OMe}t}\right) \]

where \( f \) is the fraction of dissociated S1-OMe at time \( t \), \( f_0 \) is the fraction that was not complexed prior to addition of the chase, \( f_w \) is the maximum fraction dissociated and \( k_{-1\ S1-OMe} \) is the dissociation rate constant.

Native gel-shift assays used for determining the dissociation rate constant of product oligonucleotides (k_{3\ S1-OMe}, k_{4\ S1-OMe}, k_{5\ S1-OMe} and k_{6\ S1-OMe})

The determination of product dissociation constants was attempted as described above using 5'-32P-labeled P_5 or 3'-32P-labeled P_3 in lieu of 5'-32P-labeled S1-OMe. These experiments were carried out for each product absent the other, as well as for each product in the presence of the other (50 μM).

Native gel-shift assays used for determining equilibrium dissociation constants of substrate analogs (K_d S1-OMe and K_d S1-DNA)

The 5'-32P-labeled substrate analogs S1-OMe and S1-DNA were incubated with varying concentrations of catalyst at 24°C in CB200 and 7% sucrose for 1.5 h. Aliquots were run on native gels as described above, except that the electrophoresis apparatus was maintained at 24°C. The fractions of bound substrate analog were determined and fit against the total catalyst concentration according to the hyperbolic relationship given by:

\[ \frac{[EP]}{[P] + [EP]} = \frac{[E]_T}{(K_d + [E]_T)} \]

where \([EP]/([P]+[EP])\) is the fraction of 5'-32P-labeled oligonucleotide bound to catalyst at the different total catalyst concentrations ([E]_T) and \( K_d \) is the equilibrium dissociation constant for the catalyst–substrate analog complex.

Attempts at ligation: single turnover product ligation experiments

Ligation experiments were performed by mixing equal volumes of a solution containing 2000 nM catalyst and 30 μM unlabeled P_y, and a solution containing trace 5'-32P-labeled P_y (≤1 nM) at 24°C, resulting in a final solution of 1000 nM catalyst, 15 μM 3’-product and trace quantities of the 5'-32P-labeled P_y (≤1 nM) in CB200. Aliquots were removed, quenched in the formamide solution described above and resolved on a 20% D-PAGE gel. This experiment was repeated with 1 5 μM unlabeled P_y and trace amounts of 3'-32P-labeled P_y.

Attempts at ligation: external equilibrium shift

An aliquot of 200 nM catalyst was incubated with trace quantities of 5'-32P-labeled S1 in CB200 at 24°C until the substrate had cleaved to near completion (19.5 h or 50.1 half-lives). A chase solution of unlabeled P_3 in CB200 was then added so that the final concentration of 3’-unlabeled product was 2.7 μM. A control reaction was set up where an equivalent volume of CB200 buffer minus P_y was added as the chase. Aliquots from both sets of reaction were removed, quenched in the described formamide solution and resolved on a 20% D-PAGE gel. The variation of 5'-32P-labeled S1 and 5'-32P-labeled P_y with time was monitored in attempts to measure a ligation rate.

RESULTS

The 925-11 catalytic motif was combinatorially selected as the self-cleaving 925-11c species (Figure 1A), where two modified nucleotide triphosphates were simultaneously incorporated in the selection process. Resynthesis of a portion of the self-cleaving sequence (the guide sequences and the intervening catalytic domain) on the solid phase using phosphoramidite congeners of the modified triphosphates afforded the trans-cleaving catalyst (Figure 1B) that was studied herein. The ‘standard conditions’ at which this catalyst is studied employ the same buffer used in the combinatorial selection for 925-11c (40). The products of the overnight cleavage of substrate S1 by 925-11t in CB200 are a 5’-cleavage product containing a 3’-2’ cyclic phosphodiester and a 3’-cleavage product containing a 5’-OH terminus as determined by MALDI-TOF analysis (see Supplementary Material).

The temperature at which 925-11t exhibits the fastest apparent rate for multiple turnover was chosen for this characterization. This temperature was determined through a survey of multiple turnover performed with 100 nM 925-11t catalyst and 15 μM (Figure 2) or 10 μM (data not shown) S1 at four different temperatures. As a preliminary survey used to determine the temperature at which 925-11t turns over substrate the fastest, the presence of complications such as burst or lag-phase kinetics due to factors such as product inhibition or folding were ignored. Data fit to Equation 2 gave \( k_{obs\ MTR} \) safe to a three-dimensional solution structure.
values that were divided by the catalyst concentration (100 nM) to give the rate constant \( k_{\text{cat MTR}} \), which did not differ significantly at 10 μM or 15 μM S1 at all the temperatures surveyed. Because of this invariance, the rate constants measured at 15 μM S1 were assumed to approximate \( k_{\text{cat MTR}} \), the first-order rate constant at saturating amounts of S1 and a direct measure of the cleavage ability of 925-11t. This temperature survey revealed that the optimal temperature for catalysis is near 24°C with an apparent first-order rate of 0.024 ± 0.005 min\(^{-1}\) (\( k_{\text{cat MTR}} \)) (Figure 2) and defines the temperature of the ‘standard conditions’ in which 925-11t is assayed.

The kinetics of 925-11t at 24°C

The kinetic dissection herein includes experimental values for the Michaelis constant (\( K_m \)), the rate constant for S1 dissociation (\( k_{-1} \)) and the rate constant for S1 cleavage (\( k_{\text{cat}} \)). From these values, the substrate association rate constant, \( k_1 \), was calculated. Product release rate constants (\( k_{-3}, k_{-4}, k_{-5} \) and \( k_{-6} \)) were also estimated. The minimal kinetic scheme for intermolecular cleavage of S1 under ‘standard conditions’ at 24°C is summarized in Figure 3.

**Determination of the maximum first-order rate constant at saturating catalyst concentrations, (\( k_{\text{cat STR}} \)), and the concentration of catalyst at which the reaction rate is half-maximal (\( K_{m STR} \))

The pre-steady-state kinetic constants, \( k_{\text{cat STR}} \) and \( K_{m STR} \), were determined under single turnover conditions. Data from single turnover experiments obtained at catalyst concentrations ranging from 10 to 2500 nM were fit to Equation 1 (Figure 4A). The observed pseudo-first-order constants, \( k_{\text{obs}} \), obtained from these fits were plotted against catalyst concentration in the hyperbolic Equation 6, to give values

\[
k_{\text{cat STR}} = \frac{\text{obs}}{K_{m STR} + [\text{E}]}
\]

The maximum 24°C first-order rate constant at saturating catalyst concentration, \( k_{\text{cat STR}} \), is 0.037 ± 0.001 min\(^{-1}\), and the concentration of catalyst at which the reaction rate is half-maximal, \( K_{m STR} \), is 69 ± 7 nM. From these parameters, a second-order rate constant (\( k_{\text{cat STR}}/K_{m STR} \)) of \((5.3 ± 0.5) \times 10^5 \text{ M}^{-1} \text{ min}^{-1}\) was calculated.

**Determination of the dissociation rate constant of S1 (\( k_{-1} \))

The dissociation rate constant of S1 was determined by pulse-chase experiments under single turnover conditions and confirmed by native gel experiments on the S1 substrate analog S1-OMe (\( k_{-1} \) S1-OMe). The basis for pulse-chase experiments under single turnover conditions lies in the isolation of the catalyst–S1 complex. The pulse phase allows for the formation of 32P-S1–catalyst complex. The addition of the chase molecule (excess unlabeled S1) precludes any rebinding of dissociated 32P-labeled S1. Post-chase, the 32P-S1–catalyst complex decays through two parallel pathways: substrate cleavage (governed by \( k_{\text{cat}} \)) and substrate dissociation (governed by \( k_{-1} \)). Only the results of the cleavage step can be detected by denaturing PAGE. The S1 dissociation constant, \( k_{-1} \), was determined from the observed first-order rate constant for the chased reaction, \( k_{\text{chase}} \), according to Equation 7 (4).

\[
k_{\text{chase}} = k_{\text{cat}} + k_{-1}
\]

Experimental pulse-chase data were fit to Equation 1 (Figure 5) and the rate constant of the chased reaction, \( k_{\text{chase}} \), was found to be 0.41 ± 0.05 min\(^{-1}\). The \( k_{\text{cat STR}} \) value of 0.037 ± 0.001 min\(^{-1}\) was chosen to represent \( k_{\text{cat}} \) in Equation 7, which yields a value for the dissociation rate constant for S1, \( k_{-1} \), of 0.37 ± 0.05 min\(^{-1}\).

The dissociation rate constant for the non-cleavable analog of S1, S1-OMe, was measured using native gel-shift assays using a pulse-chase technique (Figure 6) in order to confirm the value of the substrate dissociation constant \( k_{-1} \) observed in single turnover pulse-chase reactions that measured cleavage. Fitting these data to Equation 4 yielded a value of 0.44 ± 0.03 min\(^{-1}\) for the dissociation rate constant of the 2’OMe
analog of S1 from 925-11t, $k_{\mathrm{cat}}/C_0$ S1-OMe. This value is consistent with the measured value of $k_{\mathrm{cat}}/C_0$ for S1.

Gel-shift assays for equilibrium dissociation constants of catalyst–substrate analog complexes ($K_d$)

The value of $K_d$ for S1 at 24°C is an appropriate estimate of the equilibrium dissociation constant for the catalyst–substrate complex given that the dissociation rate constant, $k_{-1}$, for the substrate is much greater than the rate constant of cleavage, $k_{\mathrm{cat}}$. The $K_d$ values of the DNA and 2'OMe substrate analogs of S1 were measured to confirm the value of $K_m$ STR determined for S1 under catalyst excess conditions. The values measured by native gel-shift electrophoresis are 43 ± 9 nM for S1-DNA and 82 ± 10 nM for S1-OMe (see Supplementary Material). These values of $K_d$ derived from the non-cleavable substrate analogs agree reasonably well with the value of $K_m$ STR, measured under single turnover conditions.

Gel-shift assays for dissociation rate constants of product oligonucleotides ($k_{-3}, k_{-4}, k_{-5} \text{ and } k_{-6}$)

The dissociation rates for both products were too rapid to measure using the native gel-shift method used to determine dissociation rate constant for the substrate analog. Even without addition of chase, the 3' product–catalyst complex could not be observed in a native gel at 1 μM catalyst concentration. It was therefore not possible to measure $k_{-3}$ and $k_{-6}$. The 5' product–catalyst complex could be observed in the native gel absent the addition of chase, but complete

Figure 4. (A) Left, plots of the fraction of substrate S1 cleaved versus time at 24°C. Cleavage reactions were performed with varying concentrations of catalyst and 0.1 nM [5',32P]S1 in buffer CB200 at 24°C. The catalyst concentrations at which kinetics were observed are (closed circle) 1200 nM (open square) 150 nM (open triangle) 80 nM, (inverted open triangle) 40 nM, (open circle) 20 nM, or (open diamond) 10 nM Catalyst. (B) Right, plot of $k_{\mathrm{obs}}$ versus catalyst concentration. Rate constants obtained from the single exponential fits in (A) were fit to a hyperbolic plot against catalyst concentration. A non-linear least squares fit gave a maximum first-order rate constant, $k_{\mathrm{cat}}$ STR, of 0.037 ± 0.001 min⁻¹ and a catalyst concentration at which the reaction rate is half-maximal, $K_m$ STR, of 69 ± 7 nM. $R^2$ is 0.978. Insets show low abscissa values for the corresponding figures.

Figure 5. Determination of the substrate dissociation rate constant for substrate s1 from pulse-chase experiments at 24°C. Saturating amounts of catalyst (open square) 1800 nM and (closed circle) 1200 nM] were incubated with 0.1 nM [5',32P]S1 and run as a control for pulse-chase experiments. Multiple trials of pulse-chase experiments (open diamond, open square and open circle) were performed with 1000 nM catalyst, 0.1 nM 5'-32P-labeled S1 and 75 μM of excess unlabeled S1 as the chase. Fraction cleaved values for chased reactions have been corrected for substrate cleavage that occurred prior to the chase. Zero time for the chased reactions is referenced to the time at which the chase was added. Results of the fit for the chased reaction: $k_{\mathrm{chase}} = 0.41 ± 0.05 \text{ min}^{-1}$, amplitude = 4.0 ± 0.1%, $R^2$ is 0.849.
a dissociation was observed for even the earliest time point (30 s) following addition of the chase, making the measurement of $k_{-1}$ and $k_{-5}$ impossible (see Supplementary Material). Furthermore, the 5’ product dissociated quickly following addition of the chase even at 4°C during gel loading (data not shown). These observations suggest that dissociation of both products is probably much faster than the chemical step $k_{cat}$ at 24°C.

24°C multiple turnover kinetics

Multiple turnover kinetics was performed at varying catalyst concentrations (50–1000 nM) and saturating S1 concentrations (15 µM). Burst and lag-phase kinetics were not apparent in plots of S1 cleaved as a function of time examined at saturating concentrations of S1 at five different catalyst concentrations (Figure 7A). Linear regression analyses (Equation 2) of these data gave observed rates ($k_{obs}$ MTR) that varied directly with catalyst concentration (Equation 3) (Figure 7B). The first-order rate constant at saturating substrate conditions for multiple turnover substrate cleavage, $k_{cat}$ MTR, is 0.030 ± 0.002 min$^{-1}$. This value is very close to the maximum rate constant obtained under single turnover conditions ($k_{cat}$ STR = 0.037 ± 0.001 min$^{-1}$).

24°C substrate specificity studies

To test the specificity of 925-11t toward the nature of the unpaired nucleotide bases at or downstream from the scissile ribose, six sequence variants of S1 were synthesized and tested in a single turnover context (Table 1). First-order rate constants, $k_{cat}$ STR, were determined for each substrate at saturating catalyst concentrations. For substrates that display $k_{cat}$ values similar to those of unmodified S1, values of $k_{cat}$ STR were calculated from plots of pseudo-first-order rate constants ($k_{obs}$) at different catalyst concentrations (Equation 6). For substrates where $k_{cat}$ STR values were 10-fold lower than that of S1, catalyst saturation was confirmed by comparing $k_{obs}$ values at 5 µM and 10 µM catalyst. Because $k_{obs}$ values were similar at these concentrations, it was assumed that the measured value of $k_{obs}$ was an accurate approximation of $k_{cat}$ STR.

Substrate specificity was initially investigated by varying the base at the site of cleavage (Table 1, Entries 1–4). Substitution at this site affected a drop in $k_{cat}$ STR values by up to a factor of 50. Purine–purine and pyrimidine–pyrimidine substitutions were investigated at sites +1 and +2 from the ribose cleavage site. Of the measured substitutions, a purine–purine substitution next to the cleavage site produced the most significant rate change, while substitution of T for C at the +2 position also depressed $k_{cat}$ by more than an order of magnitude.

Effects of 1 mM Mg$^{2+}$ and of increasing EDTA on 925-11t

In order to evaluate the potential utility of modified, M$^{2+}$-independent DNAzymes for targeting mRNA in cells, the activity of 925-11t was reevaluated under physiologically relevant concentrations of Mg$^{2+}$. We measured values for $k_{cat}$ and $K_m$ under conditions where we replaced 1 mM EDTA with 1 mM Mg$^{2+}$. We chose a concentration of 1 mM Mg$^{2+}$ because of recent reports that suggest that the actual physiological concentration of free Mg$^{2+}$ is more accurately estimated at ~0.6 mM (49–52). We found that the value for $k_{cat}$ STR dropped to 0.029 ± 0.002 min$^{-1}$ in the presence of

![Figure 6](https://academic.oup.com/nar/article-abstract/32/22/6660/2375635/6660)

**Figure 6.** Pulse-chase analysis by native gel electrophoresis to determine the first-order rate constant for substrate analog dissociation, $k_{-1}$ OMe. A trace of $5'[^32P]$S1-OMe 2’OME substrate analog was incubated with catalyst (1 µM) in CB200. Following the addition of an equal volume of the chase (100 µM substrate in CB200), aliquots were taken at various times and loaded onto native PAGE gels. The fraction of substrate analog dissociated was plotted versus time for multiple experimental trials (open diamond, open square and open circle). A non-linear least squares fit gave a first-order dissociation rate constant for substrate analog dissociation, $k_{1}$ OMe, for the substrate analog S1-OMe of 0.44 ± 0.03 min$^{-1}$ and $R^2$ is 0.979.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ STR (nM)</th>
<th>$k_{cat}$ STR (min$^{-1}$)</th>
<th>$k_{relative}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’-GGGTGCCGAGTCTGGT-3’</td>
<td>69 ± 7</td>
<td>0.037 ± 0.001</td>
<td>1</td>
</tr>
<tr>
<td>5’-GGGTGCCGCAGTCTGGT-3’</td>
<td>50 ± 20</td>
<td>0.0101 ± 0.0004</td>
<td>0.27</td>
</tr>
<tr>
<td>5’-GGGTGCCGACGCTGGT-3’</td>
<td>0.001390 ± 0.00003</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>5’-GGGTGCCGAGGCTGGT-3’</td>
<td>0.000659 ± 0.00003</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>5’-GGGTGCCGCATTGGT-3’</td>
<td>0.00013 ± 0.00003</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>5’-GGGTGCCGCACCTGGT-3’</td>
<td>0.00015 ± 0.00003</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>5’-GGGTGCCGCTGCTGGT-3’</td>
<td>0.001230 ± 0.00005</td>
<td>0.03</td>
<td></td>
</tr>
</tbody>
</table>

Underscored bold type indicates specific base deviations from unmodified S1. Values of $k_{relative}$ were obtained by dividing the value of $k_{cat}$ STR obtained for other substrate analogs by the value of $k_{cat}$ STR in the first row. Bold letters denote substituted base positions relative to the cleavage site and the base downstream from the cleavage site. Although $K_m$ values were not calculated for other substrates, $k_{cat}$ values were found to be the same at both 5 µM and 10 µM catalyst suggesting full complexation of substrate, at least with regards to guide-sequence recognition.
Cleavage reactions were performed with varying concentrations of catalyst, 15 mM 5'-labeled S1 in buffer CB200 at 24°C. The concentrations of catalyst at which kinetics were observed are: (closed circle) 1000 nM (open circle) 50 nM catalyst. Data were fit to linear equations. The intercept for this value is 0.000 μM/min. The slope, k_{cat MTR}, is 0.030 ± 0.002 min⁻¹ and R² is 0.981. Inset shows low abscissa values for the corresponding figure.

Figure 7. (A) Left, Multiple turnover analyses on the cleavage of S1 at 24°C. Cleavage reactions were performed with varying concentrations of catalyst, 15 μM S1, and <1 nM 5'-labeled S1 in buffer CB200 at 24°C. The concentrations of catalyst at which kinetics were observed are: (closed circle) 1000 nM (open square) 500 nM, (open triangle) 200 nM, (inverted open triangle) 100 nM or (open circle) 50 nM catalyst. Data were fit to linear equations. The lowest R² value was 0.996. Cleavage was allowed to proceed until a maximum of 20% of the substrate was cleaved. Reactions were quenched in formamide at the times indicated on the graph. (B) Right, rate constant determination for the maximum steady-state rate constant. Values of k_{cat MTR} were plotted against catalyst concentration to obtain a steady-state rate constant at saturating substrate concentrations. The intercept for this value is 0.000 ± 0.001 μM/min. The slope, k_{cat MTR}, is 0.030 ± 0.002 min⁻¹ and R² is 0.981. Inset shows low abscissa values for the corresponding figure.

DISCUSSION

On the rate discrepancy between the cis-cleaving species and the trans-cleaving catalyst

925-11t was selected based on its ability to perform self-cleavage. The ability to perform multiple turnover was subsequently demonstrated in a ‘cut down’ experiment where the hairpin loop linking the catalytic domain to the substrate was eliminated, along with other extraneous elements of 925-11c which facilitated its selection/amplification (compare Figure 1A and B). A nearly 10-fold diminution of cleavage ability was observed as a result of the cut down of 925-11c to 925-11t. At the optimal temperature for catalytic turnover with 925-11t (24°C), the first-order multiple turnover rate constant, constant irrespective of the EDTA concentration (see Supplementary Material).

Ligation by 925-11t

1 mM Mg^{2+}, whereas that for K_m STR increased to 109 nM. If these differences reflect structural or mechanistic phenomena that result from the presence of Mg^{2+}, the effect is by no means pronounced (see Supplementary Material). In addition, measurement of the steady-state rate constant, k_{cat MTR}, for 925-11t in the presence of 2 mM Mg^{2+} produced a value of 0.037 ± 0.001 min⁻¹ (see Supplementary Material), suggesting that the observed drop in k_{cat} under single turnover conditions in the presence of 1 mM Mg^{2+} is simply due experimental error (vide infra). Finally, in order to further exclude the possibility that cleavage may have been due to contaminating amounts of a divalent metal ion, we also examined the effect of increasing the concentration of EDTA in the range of 0, 1, 4 and 10 mM under multiple turnover. As expected for true metal independence, the k_{cat} value remained unchanged, indicating that the observed drop in k_{cat} MTR is not measurable for the products of S1.

Error analysis

In general, the errors we report are generated by the software used to calculate the value in question (e.g. k_{cat} or K_m value) and generally fell within 10% for data generated on the same day. In this report, the values for k_{cat} obtained for single and multiple turnover were 0.037 min⁻¹ and 0.03 min⁻¹, respectively. A few weeks later, we repeated this work to obtain values for k_{cat} and K_m in the presence of 1 mM Mg^{2+}. As a control, we examined single turnover cleavage in the absence of Mg^{2+} and found a rate constant of 0.029 min⁻¹. This suggests that variance over time may be as high as 30%. Others have reported the same for unmodified ribozymes (45,53). Although we do not have an immediate explanation for the observed variance, we have found that use of freshly purified catalyst along with freshly labeled substrates returned slightly higher values of k_{cat}. In addition, we have recently found that the catalyst is sensitive to ultraviolet light and this may suggest other undescribed sensitivities to oxidation and radical reactions.
$k_{\text{cat}}$, is 0.03 min$^{-1}$, whereas the first-order rate constant for self-cleavage, $k_{\text{self-cleav}}$, at 24°C, for the cis-cleaving counterpart, 925-11c is 0.20 min$^{-1}$ (54). Similar diminutions have been reported for natural ribozymes when comparing rate constants of cis- and trans-cleaving species (4,53).

On the rate constants associated with the minimal kinetic model

The values of all the reported constants depicted in Figure 3 have been confirmed through complementary studies, i.e. both single and multiple turnover assays. The substrate dissociation rate constant was confirmed through pulse-chase/native gel assays, which were used to measure the dissociation rate constant of the non-cleavable substrate analog S1-OMe. Both studies show that substrate dissociation is much faster than S1 cleavage. Thus, the equilibrium dissociation constants, $K_{d_{\text{S1-OMe}}}$ and $K_{d_{\text{S1-DNA}}}$, measured for the substrate analogs could be used to confirm the value of $K_{m_{\text{STR}}}$ determined in single turnover assays. The rapid dissociation rate constant and the slow cleavage value measured for the 925-11t system made it difficult to measure the association rate constant directly using experimental precedent (46–48). The calculated 24°C association rate constant $k_1$, for S1, a 15 nt target containing a single ribose, that hypothetically formed 12 W-C base pairs, was calculated from values of $K_{m_{\text{STR}}}$ and $k_{\text{cat_{STR}}}$ and $k_1$ is $(5.9 \pm 0.7) \times 10^6$ M$^{-1}$ min$^{-1}$ and this value was verified using the calculated value of the association constant for S1-OMe $(4.4 \pm 0.4) \times 10^6$ M$^{-1}$ min$^{-1}$ from values of $K_{d_{\text{S1-OMe}}}$ and $k_{-1$ $\text{S1-OMe}}$.

Attempts to measure the product dissociation constants by pulse-chase/native gel assays were unsuccessful because the products dissociated too rapidly to be measured accurately. Nevertheless, these data suggest that the product dissociation constants are much larger than $k_{\text{cat}}$. These data are confirmed by the absence of a burst phase in multiple turnover assays and by the agreement between the value of $k_{\text{cat}}$ in catalyst excess conditions ($k_{\text{cat_{STR}}}$) and substrate excess conditions ($k_{\text{cat_{MTR}}}$).

On the importance of comparing rates obtained in catalyst excess conditions ($k_{\text{cat_{STR}}}$ and $K_{m_{\text{STR}}}$) versus substrate excess conditions ($k_{\text{cat_{MTR}}}$ and $K_{m_{\text{MTR}}}$)

Factors considered in generating the minimal kinetic model

The described single turnover kinetic assays are useful in monitoring only rates up to and including the substrate cleavage event. In general, the steady-state rate constant observed in multiple turnover kinetics will be a composite of all rate constants, including those that govern docking, conformational remodeling, chemistry and product release. In this case, the steady-state rate constant, $k_{\text{cat_{MTR}}}$, approximates the value of $k_{\text{cat_{STR}}}$. The agreement between values of $k_{\text{cat_{STR}}}$ and $k_{\text{cat_{MTR}}}$ suggests a common rate-limiting step. Slow product release is thus discounted as the rate-limiting step under multiple turnover conditions. This conclusion is also corroborated by the absence of burst phase multiple turnover kinetics, which are observed in cases of rate-limiting product release. The applicability of Equations 1, 3 and 6 in determining $k_{\text{cat_{STR}}}$ and $k_{\text{cat_{MTR}}}$ suggests the following two key points: (i) catalyst or S1 aggregates (i.e. [E$_2$S], [ES$_2$], higher order aggregates) are not kinetically active species because single turnover kinetic plots fit to Equation 6, while multiple turnover rates are directly proportional to catalyst concentrations (Equation 3); and (ii) catalyst or substrate folding steps are fast relative to cleavage as suggested by the absence of sigmoidal or lag-phase kinetics in single or multiple turnover assays (55).

A more complicated binding mechanism involving binding of S1 to catalyst followed by a conformational change [e.g. helix docking (56)] could result in a misestimate of the overall dissociation rate constant of S1 as well as an underestimation of the real value of $k_{\text{cat}}$. Nevertheless, the dissociation of S1 as observed by kinetic pulse-chase experiments and the dissociation of S1-OMe as observed by native gel-shift assays both fit to single exponential equations (Equations 1 and 4); furthermore, the rate constants obtained in these two experiments are in good agreement. It is through these observations that rate-determining conformational changes within the catalyst–substrate complex are excluded from the kinetic scheme, where a single-step binding mechanism is proposed for 925-11t under standard conditions.

On the substrate dissociation rates, association rates and catalytic efficiency of a modified nucleotide catalyst

Values for constants of substrate association, dissociation and catalytic efficiency have been compiled for different DNAzymes and ribozymes in Table 2, such that a comparison can be made between 925-11t and other well-known Mg$^{2+}$-dependent systems that display turnover (4,12,45,53,57–59). The second-order rate constants for the association of S1 with 925-11t, $k_1$, lies at the lower range expected for substrate association. This deviation is not extraordinary as the $k_1$ for 925-11t lies within an order of magnitude of 8–17cb and that of the natural hairpin and HDV systems. The relatively large rate for the dissociation of S1 from 925-11t, $k_{-1}$, is not unusual, as this value is equal to that of DNAzyme systems and falls between that of the HDV and hairpin ribozymes constructs.

On ligation

The naturally occurring hammerhead (45) and hairpin (53) ribozymes as well as the combinatorially selected 10–23 DNAzyme (12) catalyze ligation. The hepatitis delta virus ribozyme does not facilitate a measurable rate of ligation (Table 2, Entry 6). One argument for the absence of ligation in the HDV ribozyme is that the dissociation constants for the products are very fast (dissociation rate for the $S^-$product $> 12$ min$^{-1}$) (4). It can be assumed that the reverse reaction in the internal equilibrium, $k_{-2}$, does occur, however, the rate at which this happens is much slower than the release of products from the enzyme–product complex. Another explanation forwarded by Ke et al. (60) suggests that the HDV ribozyme undergoes a significant conformational change after RNA cleavage that prevents ligation. Either of rapid product release or of conformational change upon cleavage could account for our inability to observe ligation with 925-11t.

On 925-11t substrate specificity

Substrate specificity of 925-11 was initially investigated with regard to the base on the scissile ribose (Table 1, Entries 1–4).

Interestingly, a C to U substitution results in a 25-fold drop in rate, a C to G substitution results in a 50-fold drop in rate and a C to A substitution results in a 4-fold drop in rate. These results suggest that the exocyclic amine present on both A and C (and absent on U or G) and/or the hydrogen acceptor ability at the N1 adenosine or the N3 cytosine positions are critical for recognition.

Common to the 9_25-11, 10–23, 8–17 and Bipartite DNAzymes is the fact that the scissile ribonucleotide is not Watson–Crick paired. The 10–23 DNAzyme displays a similar level of sequence specificity to 9_25-11 at this residue; cleavage was observed to favor, in order of decreasing preference, G, A, U and C (12). The G to A substitution was well tolerated with a decrease in rate of a factor of 3; however, substitution of a pyrimidine for the purine dropped the value of $k_{cat}$ by greater than a factor of 25 highlighting the importance of a guanine at the cleavage site in the case of the 10–23 DNAzyme. The 8–17 DNAzyme recognizes the unpaired base on the scissile ribose with limited selectivity as similar cleavage rates are observed for any 5'-rNG-r3' target (13). In general, the unpaired state of at least one of the bases flanking the scissile ribophosphodiester linkage may be a characteristic feature of many RNA-cleaving DNAzymes and RNAzymes where a ‘looped-out’ base appears to be integral to positioning the 2'-OH for an in-line attack on the phosphate (61,62).

For 9_25-11t, substitution at the +1 position on S1 directly downstream of the scissile residue (Table 1, Entry 5) produced the largest drop in the value of $k_{cat}$ (150-fold), implying that the characteristics of the guanine at this position are also vital for the activity of 9_25-11t. Substitution at the +2 position downstream also resulted in a 12-fold drop in the value of $k_{cat}$. (Table 1, Entry 7). The substitution at this position has a similar effect on $k_{cat}$ relative to U or G substitutions on the scissile ribose; however, the effect of this substitution is not as detrimental to the activity of 9_25-11t as a substitution +1 to the cleavage site. Substitution at the +2 position of S1 does not have a cumulative effect with a purine substitution at the +1 position (Table 1, Entry 6).

<table>
<thead>
<tr>
<th>Entry</th>
<th>Ribozyme (construct)</th>
<th>Conditions</th>
<th>Substrate dissociation constant, $K_d$ (10$^{-6}$ M)</th>
<th>Substrate turnover constant, $k_{cat}$ (10$^{-3}$ M$^{-1}$ s$^{-1}$)</th>
<th>Substrate cleavage constant, $k_{cat}/K_d$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9_25-11t</td>
<td>200 mM NaCl, pH 7.5, 20°C</td>
<td>5.9 0.37 0.037 0.037</td>
<td>5 15 (7/5)</td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10–23</td>
<td>2 mM MgCl$_2$, pH 7.5, 37°C</td>
<td>14 0.21 0.011 0.011</td>
<td>20–100 (8/7)</td>
<td>(57)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>8–17</td>
<td>5 mM MgCl$_2$, pH 7.5, 25°C</td>
<td>16 0.003 0.01 0.011</td>
<td>18 (8/8)</td>
<td>(45)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Hammerhead (HH16)</td>
<td>10 mM MgCl$_2$, pH 7.4, 25°C</td>
<td>270 0.001 0.003</td>
<td>100 5 (4/6)</td>
<td>(53)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Hairpin (SV5)</td>
<td>12 mM MgCl$_2$, pH 8.0, 37°C</td>
<td>21 0.001 0.0001</td>
<td>5 5 (6/8)</td>
<td>(58)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>7 Group II intron</td>
<td>100 mM MgCl$_2$, pH 7.5, 45°C</td>
<td>270 0.004 0.01</td>
<td>200 5 (0/6)</td>
<td>(58)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>VS (G11/Ava S)</td>
<td>5 mM MgCl$_2$, pH 8.0, 30°C</td>
<td>&lt;0.01 0.16 0.13</td>
<td>200 5 (0/6)</td>
<td>(69)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>VS (G11/Ava S)</td>
<td>5 mM MgCl$_2$, pH 8.0, 30°C</td>
<td>2 mM spermidine, 30°C</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

Entries 1–3 are DNAzymes, while entries 4–8 are ribozymes. Where multiple literature reports exist, rate constants are reported at lowered Mg$^{2+}$ concentrations. * indicates calculated or inferred values.
contribute to an increase in the apparent value of $k_{\text{cat}}$ based on contextual positioning within a secondary/tertiary structure (64,65).

**Comparisons with other catalytic nucleic acids**

The first-order rate constants for ribophosphodiester cleavage for 925-11t are comparable (within one log) with those of other M$^{2+}$-dependent ribozyme systems that have been characterized in detail with regards to turnover at low millimolar Mg$^{2+}$ concentrations. By covalently modifying DNA with imidazole and cationic amine functional groups normally displayed on proteins, a respectable rate of cleavage is obtained in the absence of a M$^{2+}$. Furthermore, these modifications do not greatly perturb the constants associated with oligonucleotide substrate or product binding and release when compared with other DNAzyme and RNAzyme models (Table 2).

The combinatorially selected cis-cleaving modified DNA sequence, 925-11c, displays a 24$^\circ$C ribophosphodiester cleavage rate, $k_{\text{self-cleave}}$, of 0.20 min$^{-1}$, representing a 10$^2$ to 10$^4$-fold improvement over M$^{2+}$-independent, unmodified self-cleaving DNA counterparts that display first-order rate constants on the order of 10$^{-3}$–10$^{-4}$ min$^{-1}$ (18–20). Admittedly, the value for $k_{\text{self-cleave}}$ at 24$^\circ$C for 925-11c lies at the lower end of the spectrum when compared with many natural ribozymes. For example, the cleavage rate constant at 37$^\circ$C is 36 min$^{-1}$ for the RNA component of Bacillus subtilis tRNA$^{\text{Asp}}$ RNAse P (66). A variant of the Varkud Satellite ribozyme displays an extraordinary self-cleavage values upwards of 600 min$^{-1}$ at 24$^\circ$C in the presence of 400 mM Mg$^{2+}$ (67).

However, consideration of some of the extraordinary $k_{\text{cat}}$ values for self-cleaving systems is somewhat tangential to a discussion of catalysis with respect to the trans-cleaving systems listed in Table 2. When trans-cleaving variants of these ribozymes are engineered (e.g. VS), the steady-state rate constant for cleavage is often limited by either substrate association or slow folding rate constants prior to cleavage and low rate constants for product release following cleavage (59,68).

Indeed, 925-11t cleaves within the same order of magnitude of trans-cleaving ribozymes and DNAzymes that have been characterized in detail (Table 2). In fact at physiological ionic strength, 925-11t fares better than the combinatorially selected 8–17c sequence which displays rate constants of 10$^{-2}$–10$^{-1}$ min$^{-1}$ at 3 mM Mg$^{2+}$ (69). Nevertheless, at elevated M$^{2+}$ concentrations, both 10–23 and 8–17 display values for $k_{\text{cat}}/K_m$ that are 1–3 orders of magnitude greater than ours. Furthermore, in the case of 925-11t, addition of Mg$^{2+}$ did not improve $k_{\text{cat}}/K_m$. In addition, the activity of 925-11t was significantly diminished at 37$^\circ$C, suggesting a further limitation that must be overcome for physiological use.

Despite both a generally lower $k_{\text{cat}}$ and a reduced catalytic efficiency that is evident with 925-11t when compared with other metal dependant DNAzymes and ribozymes (Table 2), RNA cleavage in the absence of a M$^{2+}$ or other co-factor is an especially salient feature in underscoring the potential for using modified bases to cleave RNA targets at physiological concentrations of Mg$^{2+}$. This aspect of 925-11t not only distinguishes this catalyst from the listed nucleic acid catalysts but also suggests a different mechanism of ribozyme cleavage invoking substrate distortion through extended non-canonical base pairing.

This modified catalyst, 925-11t, as a first-in-class prototype RNAs E A mimic, displays many similarities to the initially reported M$^{2+}$-dependant DNAzymes (70,71). For instance, as with the early DNAzymes that were selected to cleave at a single embedded ribose but would not cleave all RNA target, 925-11t only cleaves a substrate with one embedded ribose with similar rates and efficiencies. As a proof of concept for M$^{2+}$-independent ribophosphodiester hydrolysis, the demonstration of using synthetic functionalities to target RNA with DNAzymes alleviates concerns regarding the disputed intracellular availability of divalent metal cations. Reselection with the triphosphates in Figure 1C should yield dually modified DNAzymes that will cleave all RNA targets. Indeed, Sidorov et al. (41) have already demonstrated self-cleavage of an all RNA target by modified DNA sequence selected from similarly modified triphosphates. Together with ours, such work portends a rich and varied approach to using modified selections for the development of RNA cleavage reagents.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at NAR Online.

**ACKNOWLEDGEMENTS**

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