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RNA-Cleaving DNA Enzymes with Altered Regio- or Enantioselectivity

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Abstract: In vitro evolution methods were used to obtain DNA enzymes that cleave either a 2′,5′-phosphodiester following a D-ribonucleotide or a 3′,5′-phosphodiester following an L-ribonucleotide. Both enzymes can operate in an intermolecular reaction format with multiple turnover. The DNA enzyme that cleaves a 2′,5′-phosphodiester exhibits a k_{cat} of ~0.01 min^{-1} and catalytic efficiency, k_{cat}/K_m, of ~10^8 M^{-1} min^{-1}. The enzyme that cleaves an L-ribonucleotide is about 10-fold slower and has a catalytic efficiency of ~4×10^5 M^{-1} min^{-1}. Both enzymes require a divalent metal cation for their activity and have optimal catalytic rate at pH 7−8 and 35−50 °C. In a comparison of each enzyme's activity with either its corresponding substrate that contains an unnatural ribonucleotide or a substrate that instead contains a standard ribonucleotide, the 2′,5′-phosphodiester-cleaving DNA enzyme exhibited a regioselectivity of 6000-fold, while the L-ribonucleotide-cleaving DNA enzyme exhibited an enantioselectivity of 40-fold. These molecules demonstrate how in vitro evolution can be used to obtain regio- and enantioselective catalysts that exhibit specificities for nonnatural analogues of biological compounds.

Introduction

Two important properties of an enzyme are its catalytic rate enhancement and its specificity for a particular chemical transformation. There are several kinds of specificity, including that for a particular class of substrate, that for a particular regioisomer, and that for a particular stereoisomer. Naturally occurring enzymes, composed of either proteins or nucleic acids, exhibit these various types of selectivity as a consequence of their complex structure. Even simple peptides or oligonucleotides are capable of operating with a high degree of specificity.[1−3]

The ability to obtain novel protein and nucleic acid enzymes through directed evolution has enabled the development of artificial enzymes that are capable of performing regio- or stereoselective chemical reactions.[4−7] Evolved protein enzymes have found industrial applications as chiroselective catalysts.[8] RNA enzymes have been obtained that catalyze a Diels−Alder cycloaddition reaction and operate with an enantiomeric excess (ee) of greater than 95%.[9] When that same RNA enzyme was prepared from L- rather than D-nucleotides, it of course produced the opposite enantiomeric product with the same ee value.

Nucleic acid enzymes also have been shown to operate in a regiospecific manner. For example, the class I RNA ligase ribozyme selectively catalyzes the formation of a 3′,5′- rather than 2′,5′-phosphodiester linkage.[10,11] The hammerhead ribozyme cleaves 3′,5′- but not 2′,5′-phosphodiester linkages of RNA.[12] The hepatitis delta virus ribozyme preferentially cleaves the natural 3′,5′-linkage, but also operates with about 100-fold-reduced rate in cleaving a 2′,5′-phosphodiester.[12]

To explore further the regio- and enantiospecificity of nucleic acid enzymes, this study employed in vitro evolution methods to isolate two novel DNA enzymes, one that cleaves a 2′,5′-linked β-D-ribonucleotide and another that cleaves a 3′,5′-linked β-L-ribonucleotide (Figure 1A). The in vitro evolution experiments began with separate populations of ~10^{15} random-sequence DNA molecules, and each gave rise to Mg^{2+}-dependent DNA enzymes that performed the target reaction. Both enzymes were made to operate on a separate nucleic acid substrate with multiple turnover. The 2′,5′-phosphodiester-cleaving DNA enzyme exhibited a rate enhancement of about 20 000-fold compared to the uncatalyzed reaction, whereas the L-ribonucleotide-cleaving DNA enzyme exhibited a catalytic rate enhancement of about 500-fold. The former operated with a regioselectivity of about 6000-fold, while the latter operated with an enantioselectivity of about 40-fold.

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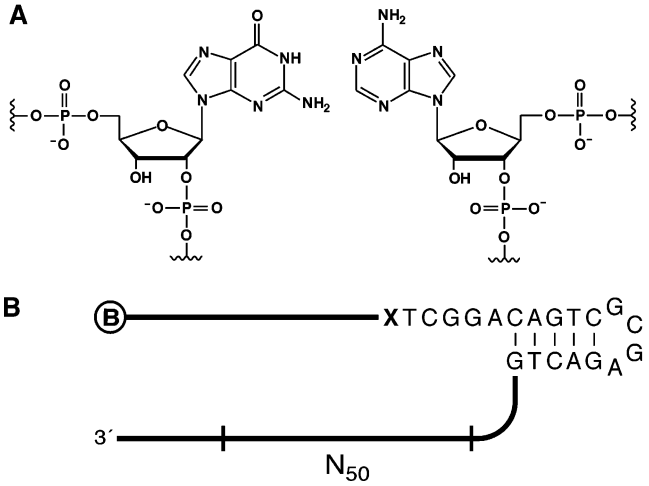

Figure 1. Compounds employed in the development of DNA enzymes that cleave unnatural ribonucleotide analogues: (A) chemical structure of a 2′,5′-linked β-D-guanylate (left) and 3′,5′-linked β-L-adenylate (right); (B) starting library of DNA molecules that were used to obtain DNA enzymes with the desired activity. Each molecule contained a 5′-terminal biotin (encircled B), either a 2′,5′-linked D-nucleotide or 3′,5′-linked L-nucleotide at the target cleavage site (X), a fixed hairpin region downstream from the cleavage site (sequence shown), and 50 random-sequence deoxynucleotides (N_{50}).

Experimental Section

Chemical Synthesis of Oligonucleotides. β-D-3′-*tert*-butyldimethylsilyl- and β-L-2′-*tert*-butyldimethylsilyl-ribonucleoside phosphoramidites were obtained from ChemGenes (Ashland, MA), and all other nucleoside phosphoramidites were from Glen Research (Sterling, VA). All oligonucleotides were prepared by automated synthesis using an Applied Biosystems Expedite nucleic acid synthesizer. A 15-min coupling step was employed for the 3′-*tert*-butyldimethylsilyl- and L-ribonucleoside phosphoramidites. The resulting oligonucleotides were deprotected by incubation in anhydrous saturated NH_3/ethanol for 36 h at 37 °C, followed by an overnight incubation at room temperature in a solution of 1 M tetrabutylammonium fluoride in THF. All other oligonucleotides were synthesized and deprotected using standard procedures. All oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis (PAGE) and desalted on a NAP-25 column (Pharmacia Biotech, Picataway, NJ).

In Vitro Selection. Starting pools of $\sim 10^{15}$ DNA molecules were constructed by extension of 4 nmol of 5′-biotin-d(TTTTAGAGACGATGACGATGCAXTCGGACAGTCGCGAGACTG)- 3′ (primer **1**; X = 2′,5′-rG or L-rA) on 6 nmol of 5′-d(GTGCCAAGCTTACCG-N_{50}-CAGTCTCGCGACTGTCCGA)-3′ (N = A, C, G, or T; complementary sequences underlined). The 1-mL reaction mixture contained 5 units μL^{-1} Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD), 3 mM $MgCl_2$, 75 mM KCl, 50 mM tris(hydroxymethyl)aminomethane (Tris, pH 8.3), and 0.25 mM each of dATP, dGTP, dCTP, and TTP. The extension reaction was performed by annealing the two oligonucleotides at 85 °C for 4 min, cooling to room temperature, and then adding $MgCl_2$ and reverse transcriptase and incubating at 37 °C for 1 h. The extension products were purified by nondenaturing PAGE, eluted from the gel, precipitated with ethanol, and then dissolved in a 1-mL solution containing 1−2 μM extension product, 0.5 M NaCl, 0.2 mM Na_2EDTA, and 50 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-3-propanesulfonic acid (EPPS, pH 7.0). This material was applied to an affinity column containing 300 μL of UltraLink Immobilized Streptavidin PLUS gel (Pierce, Rockford, IL) that had previously been equilibrated with four 400-μL volumes of wash buffer (0.5 M NaCl, 0.1 mM Na_2EDTA, 50 mM EPPS (pH 7.0)). The column was rinsed with five 400-μL volumes of wash buffer, five 400-μL volumes of ice-cold 0.1 N NaOH/150 mM NaCl, and five 400-μL volumes of wash buffer at 37 °C and then eluted over 1 h at 37 °C with three 300-μL volumes of reaction buffer (10 mM $MgCl_2$, 0.5 M NaCl, 50 mM EPPS (pH 7.5)). Molecules that eluted from the column were precipitated with ethanol in the presence of 150 pmol of the primer 5′-d(TCGGACAGTCGCGAGACTG)-3′ (primer **2**) and 250 pmol of the primer 5′-d(AACAACAACYYYGTGCCAAGCTTACCG)-3′ (primer **3**; Y = abasic nucleotide analogue) and then PCR amplified in a 500-μL volume. The three abasic analogues created a stop site for *Taq* polymerase, which caused one of the PCR product strands to be 12 nucleotides shorter than the other.

The amplified products were precipitated with ethanol, and the longer of the two strands was isolated by denaturing PAGE, eluted from the gel, and again precipitated with ethanol. Half of the eluted DNA (~80 pmol) then was used in a template-directed extension reaction employing 200 pmol of primer **1**, under the same conditions as described above. In this and all subsequent rounds of selective amplification, the extension products were immobilized on 50 μL of Streptavidin Plus gel, rinsed with five 200-μL volumes of wash buffer, five 200-μL volumes of ice-cold 0.1 N NaOH/150 mM NaCl, and five 200-μL volumes of wash buffer at 37 °C, and then eluted with three 40-μL volumes of reaction buffer over 1 h. During round 2 the reacted molecules additionally were selected on the basis of their electrophoretic mobility in a denaturing polyacrylamide gel. During rounds 7−10 the reaction buffer was changed to 5 mM $MgCl_2$, 0.2 M NaCl, and 50 mM EPPS (pH 7.5), and the reaction time was reduced to 30 min for round 7, 5 min for round 8, and 1 min for rounds 9 and 10. Following round 10, random mutations were introduced by hypermutagenic PCR.[13] Another five rounds of selective amplification were carried out, changing the reaction buffer to 5 mM $MgCl_2$, 0.15 M NaCl, and 50 mM EPPS (pH 7.5) and reducing the reaction time to 0.5 min for round 11 and to no more than the time required for elution for rounds 12−15.

Analysis of Individual Clones. Following the 10th and 15th rounds, the DNA molecules were amplified by PCR using primer **2** and a truncated version of primer **3** having the sequence 5′-d(GTGCCAAGCTTACCG)-3′. The PCR products were cloned using the TA cloning kit and INVαF′ competent cells (Invitrogen, Carlsbad, CA). Individual colonies were isolated on agar plates and amplified by either colony PCR or inoculation of 2-mL cultures. The DNA was isolated and sequenced by the dideoxy chain termination method.[14] Cleavage assays were performed under similar conditions to those employed during in vitro selection. The reactions were quenched by the addition of an equal volume of a mixture containing 10 M urea and 50 mM Na_2EDTA, and the reaction products were separated by denaturing PAGE and analyzed using a Molecular Dynamics Phosphorimager.

Kinetic Analysis. Intermolecular cleavage reactions were carried out in the presence of 25 mM $MgCl_2$, 150 mM NaCl, and 50 mM EPPS (pH 7.5) at 37 °C. The reactions were initiated by the addition of substrate to enzyme, each contained within a mixture corresponding to the final reaction buffer. The reaction products were separated by denaturing PAGE and analyzed using a Molecular Dynamics Phosphorimager. Values for k_{obs} were obtained under single-turnover (enzyme excess) conditions, employing various concentrations of enzyme and a trace of [5′-^{32}P]-labeled substrate. Experimental data for the L-ribonucleotide-cleaving DNA enzyme were fit to a single-exponential equation:

$$F_t = F_\infty(1 - e^{k_{obs}t}) + F_0$$

Here F_t is the fraction cleaved at time t, F_∞ is the fraction cleaved at the maximum extent of the reaction, and F_0 is the fraction cleaved at

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time zero. Experimental data for the 2′,5′-ribonucleotide-cleaving DNA enzymes were fit to a double-exponential equation:

$$F_t = F_1(1 - e^{k_{obs1}t}) + F_2(1 - e^{k_{obs2}t}) + F_0$$

Here F_t is the fraction cleaved at time t, F_1 and F_2 are the amplitudes of the two phases of the reaction, k_{obs1} and k_{obs2} are the corresponding rates of each phase, and F_0 is the fraction cleaved at time zero. These parameters were estimated by nonlinear regression using the Levenberg−Marquardt algorithm (Deltagraph 4.5, SPSS Science).

Values for k_{obs} also were obtained under multiple-turnover (substrate excess) conditions for a range of concentrations of substrate that spanned K_m. Data were obtained over the first ∼15% of the reaction and were fit to a line typically based on six data points. The parameters k_{cat} and K_m were obtained from a standard Michaelis−Menten saturation plot consisting of 11−21 data points, always with [S] in at least 10-fold excess over [E] and [E] at least 5-fold below the K_m. The data were adjusted to take into account the maximum extent of the reaction for the L-ribonucleotide-cleaving DNA enzymes and the amplitude of the first phase of the reaction for the 2′,5′-ribonucleotide-cleaving DNA enzyme. Standard error values were calculated using SigmaPlot (SPSS Science).

The uncatalyzed rate of cleavage was determined by incubating 1 nM [5′-^{32}P]-labeled substrate under standard reaction conditions. Aliquots were taken over a 5-day period and analyzed by denaturing PAGE. The value for k_{uncat} was obtained from the slope of a best-fit line of the fraction cleaved versus time.

Metal, Temperature, and pH Dependence. All k_{obs} values were obtained under single-turnover conditions employing 90 nM enzyme and 1 nM [5′-^{32}P]-labeled substrate, which were incubated under standard reaction conditions as described above. $MgCl_2$ dependence was assessed over a range of $MgCl_2$ concentration of 1−100 mM for the 2′,5′-ribonucleotide-cleaving DNA enzyme and 0.1−30 mM for the L-ribonucleotide-cleaving DNA enzyme. Metal ion requirements were tested using 10 mM M^{2+}, except Pb^{2+} which was tested at 1 mM concentration. Temperature dependence was measured over a range of 10−65 °C, employing a temperature block and heated lid to control evaporation at the elevated temperatures. The pH dependence was assessed over a range of 6.0−9.5, employing three different buffers: 2-morpholinoethanesulfonic acid (MES) for pH 6.0−7.0, EPPS for pH 7.0−8.5, and 2-(cyclohexylamino)ethanesulfonic acid (CHES) for pH 8.5−9.5.

Characterization of Cleavage Products. Large-scale reactions were carried out, employing 1 nmol of either 2′,5′- or L-ribonucleotide-containing substrate, with 1 nmol of the corresponding DNA enzyme under the standard reaction conditions described above. The reactions were quenched after 24 h, and the cleavage products were purified by denaturing PAGE. Prior to the reaction, the L-ribonucleotide-containing substrate was 5′-phosphorylated using T4 polynucleotide kinase and ATP. This permitted separation of the two 9mer cleavage products on the basis of their differing electrophoretic mobility. The gel-purified products were desalted on a Nensorb-20 column (NEN Life Sciences) and analyzed by MALDI-TOF mass spectrometry, using a PerSeptive Biosystems Voyager-STR mass spectrometer.

Results

In Vitro Selection. Two separate libraries of ∼10^{15} DNA molecules each were constructed, one containing a single 2′,5′-linked guanylate and the other a single L-adenylate embedded within an otherwise all-DNA sequence. The libraries were constructed by primer extension, using a 5′-biotinylated primer that contained the unnatural ribonucleotide linkage. The primer was hybridized to a DNA template that contained 50 random-sequence deoxynucleotides flanked by residues of defined sequence that served as primer binding sites. Similar to a strategy that has been employed previously,[15] a DNA hairpin was engineered into the pool of molecules to favor base-pairing interactions surrounding the target ribonucleotide analogue (Figure 1B).

Primer extension was carried out employing reverse transcriptase as a DNA-dependent DNA polymerase to generate a double-stranded product. DNA-catalyzed cleavage could not occur during the primer extension reaction because the two strands were maintained in duplex form. The full-length, double-stranded product was purified by nondenaturing PAGE and quantified on the basis of its UV absorbance. The purified material was immobilized on a streptavidin-containing solid support, and the nonbiotinylated strand was removed by brief washing with an ice-cold solution of 0.1 N NaOH. The biotinylated single-stranded molecules that remained bound to the support then were challenged to cleave the embedded ribonucleotide linkage, thereby becoming released from the support. Initially, the reaction conditions were chosen to favor duplex formation, with high salt concentrations of 10 mM $MgCl_2$ and 500 mM NaCl at pH 7.5 and 37 °C. The released molecules were collected and amplified by PCR, thus enriching the population with reactive molecules.

A total of 15 rounds of selective amplification were performed to obtain the most active catalysts. During the first six rounds, the reaction conditions were as described above, with a reaction time of 1 h. During rounds 7−10, the reaction conditions were changed to 5 mM $MgCl_2$ and 200 mM NaCl at pH 7.5 and 37 °C. During the 7th and 8th rounds, the time allowed for the reaction was reduced to 5 min to increase the stringency of selection; during the 9th and 10th rounds the time was further reduced to 1 min. Individual molecules were cloned from the population following the 10th round and were sequenced and tested for catalytic activity. The population then was randomly mutagenized at a frequency of ∼10% per nucleotide position, and five additional rounds of selective amplification were carried out, employing reaction conditions of 5 mM $MgCl_2$ and 150 mM NaCl at pH 7.5 and 37 °C. The amount of time allowed for the reaction was reduced from 0.5 min for round 11 to no more than the amount of time required for elution during rounds 12−15. Individuals again were cloned from the population and sequenced, revealing a high degree of sequence similarity within the formerly random-sequence region (see Supporting Information).

Identification of Catalytic Motifs. One of the cloned individuals isolated following the 10th round of selection for 2′,5′-phosphodiester-cleaving activity had especially high activity and was chosen for further study. It was designated as "2′:10-16", referring to the fact that it was the 16th clone isolated following the 10th round. Analysis of its sequence suggested a plausible secondary structure, as shown in Figure 2A. The enzyme and substrate strands were prepared separately by extending the regions of presumed base pairing surrounding the cleavage site and repairing any base mismatches. The cloned individuals isolated following the 15th round of selection for 2′,5′-phosphodiester-cleaving activity had approximately the same level of activity as the 2′:10-16 clone. A high degree of sequence similarity was noted among the clones isolated following round 15 (see Supporting Information). A representative clone, designated "2′:15-2", was chosen for further analysis.

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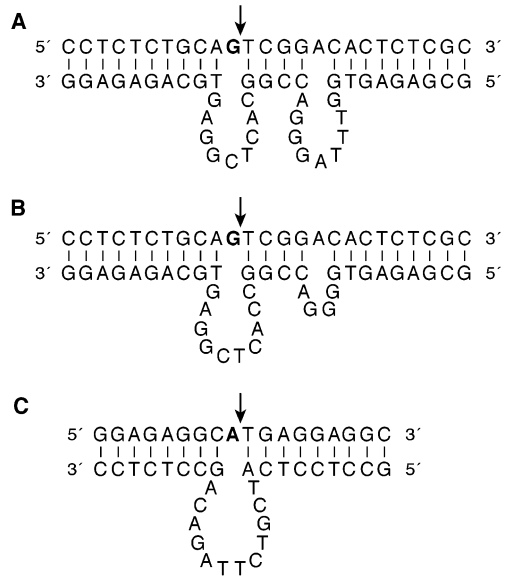

Figure 2. Putative secondary structure of the (A) 2′:10-16, (B) 2′:15-2, and (C) L:15-30 DNA enzymes, each shown bound to its substrate in the intermolecular reaction format. Bold letter **G** or **A** indicates a 2′,5′-linked β-D-guanylate or 3′,5′-linked β-L-adenylate, respectively. The arrow indicates the cleavage site.

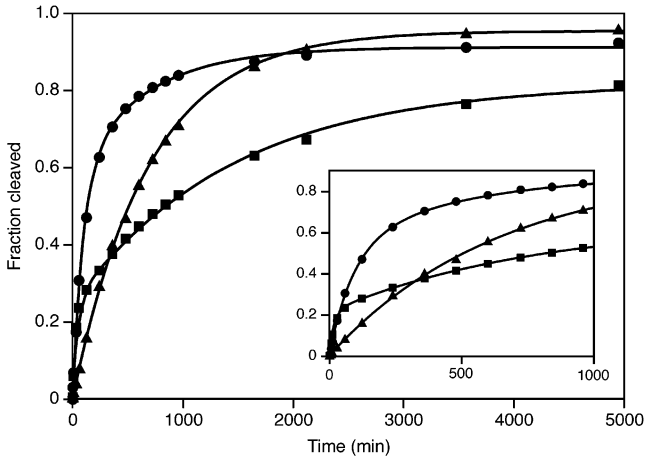

Figure 3. Time course of the cleavage reaction catalyzed by the 2′:10-16 (●), 2′:15-2 (■), and L:15-30 DNA enzymes (▲), measured under single-turnover conditions. The inset shows a detail of the first 1000 min of the reaction, demonstrating its biphasic nature for the two 2′,5′-phosphodiester-cleaving enzymes. Data were fit to either a single- or double-exponential equation (see Experimental Section). Reaction conditions: 25 mM $MgCl_2$; 150 mM NaCl; pH 7.5; 37 °C.

It was prepared by chemical synthesis, separating the enzyme and substrate strands so that cleavage would occur in an intermolecular reaction format (Figure 2B). As will be discussed in more detail below, the 2′:10-16 and 2′:15-2 DNA enzymes were able to cleave a separate substrate with multiple turnover at a rate of $\sim$0.01 min^{-1}.

In an effort to confirm the proposed secondary structure of the 2′,5′-phosphodiester-cleaving DNA enzymes, a variety of nucleotide substitutions and deletions were made within the central unpaired region, most of which resulted in a complete loss of catalytic activity. The putative stem regions on either side of the two internal bulge loops were shown to be interchangeable with any base-paired nucleotides with little or no effect on the catalytic rate. When the internal bulge loop that lies furthest from the cleavage site was replaced by a single T residue, forming a continuous stretch of base pairs downstream from the cleavage site, catalytic activity was abolished. However, the sequence of this internal bulge loop could be altered somewhat, with 3′-AGGGATTTG-5′ (Figure 2A), 3′-AGGG-5′ (Figure 2B), 3′-AGGGATTCG-5′, and 3′-AGGGATTG-5′, all resulting in full catalytic activity. Finally, the unpaired G residue located immediately upstream of the cleavage site could be changed to an A with only a slight reduction in activity but when changed to either a C or U resulted in a complete loss of activity.

The cloned individuals isolated following the 15th round of selection for L-ribonucleotide-cleaving activity were much more active compared to those isolated following the 10th round. There again was a high degree of sequence similarity among the clones isolated after the final round (see Supporting Information). A representative clone, designated "L:15-30" was chosen for further analysis. It was prepared by chemical synthesis, separating the enzyme and substrate strands and extending the regions of presumed base pairing surrounding the cleavage site while repairing any base mismatches (Figure 2C). As with the 2′,5′-phosphodiester-cleaving DNA enzymes, the base-paired nucleotides surrounding the cleavage site could be replaced by any paired nucleotides with little or no effect on the catalytic rate. No further mutational analysis was carried out on the L-ribonucleotide-cleaving motif.

Biochemical Properties of the DNA Enzymes. The catalytic properties of all three of the above-described DNA enzymes were studied in the intermolecular reaction format (Figure 2A–C). Time-course experiments revealed that the 2′:10-16 and 2′:15-2 DNA enzymes exhibited biphasic kinetics, with a fast initial rate, followed by a slower second phase of the reaction (Figure 3). Consequently, the data were fit to a double-exponential equation and the catalytic rate constant was determined for each phase of the reaction (see Experimental Section). The L:15-30 DNA enzyme, in contrast, exhibited monophasic kinetics that fit well to a single exponential (Figure 3).

Under multiple-turnover conditions, product release was shown to be rate limiting for the 2′:10-16 and 2′:15-2 DNA enzymes. This was evident by comparing the multiple-turnover reaction with a single-turnover reaction carried out under conditions of enzyme excess, the latter being about 10-fold faster. The regions of base pairing between the enzyme and substrate were shortened on either or both sides of the cleavage site to favor product release. However, product release remained rate limiting until the paired regions were made so short that catalytic activity became impaired (data not shown). The highest value for k_{cat} was obtained when the 2′:10-16 enzyme was shortened by one base pair at each end of the enzyme–substrate complex. Under multiple-turnover conditions, this construct exhibited a k_{cat} of 0.0036 $\pm$ 0.0001 min^{-1} and K_m of 0.21 $\pm$ 0.03 nM (Figure 4B). By comparison, the full-length construct exhibited a k_{cat} of 0.0022 $\pm$ 0.0001 min^{-1} and K_m of 0.042 $\pm$ 0.008 nM (Figure 4A). Under conditions of enzyme excess, the rate of cleavage for the full-length 2′:10-16 enzyme was 0.011 $\pm$ 0.0004 min^{-1} (Figure 5A), 5-fold higher than that obtained under multiple-turnover conditions. The 2′:15-2 enzyme had a slightly faster rate than the 2′:10-16 enzyme under both single- and multiple-turnover conditions. The k_{cat} of the 2′:15-2 enzyme was 0.012 $\pm$ 0.0004 min^{-1} and K_m was 0.064 $\pm$ 0.009 nM

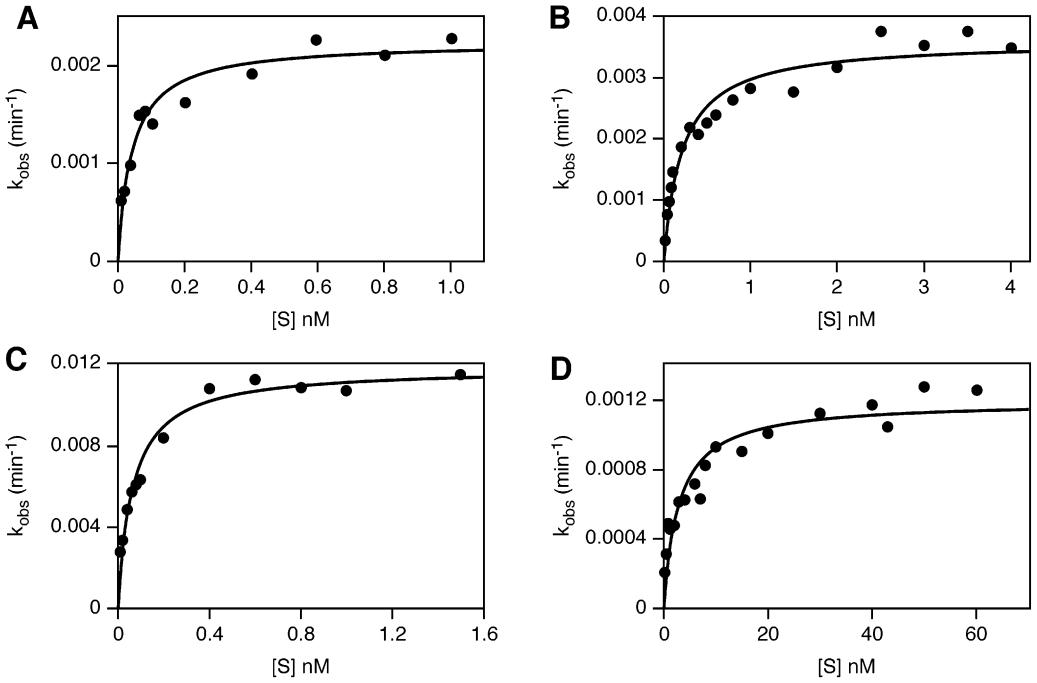

Figure 4. Catalytic activity of DNA enzymes that cleave unnatural ribonucleotide analogues, measured under multiple-turnover conditions: (A) 2′:10-16 DNA enzyme with full-length stem regions surrounding the cleavage site; (B) 2′:10-16 DNA enzyme with the stem regions shortened by one base pair each; (C) 2′:15-2 DNA enzyme; (D) L:15-30 DNA enzyme. Data were fit to a curve based on the Michaelis−Menten equation: $v = k_{cat}[\text{substrate}]/(K_m + [\text{substrate}])$. Reaction conditions: 25 mM $MgCl_2$; 150 mM NaCl; pH 7.5; 37 °C.

(Figure 4C). This corresponds to a catalytic efficiency, k_{cat}/K_m, of $\sim 10^8$ M^{-1} min^{-1}. Under conditions of enzyme excess, the rate of the 2′:15-2 enzyme was 0.034 ± 0.001 min^{-1} (Figure 5B), which is about 3-fold higher than that obtained under multiple-turnover conditions.

The regions of base pairing between the L:15-30 DNA enzyme and its substrate could be shortened so that product release was not rate limiting and without causing a reduction of the catalytic rate. Under multiple-turnover conditions, the shortened enzyme exhibited Michaelis−Menten saturation kinetics, with a k_{cat} of 0.0012 ± 0.0001 min^{-1} and K_m of 2.9 ± 0.8 nM (Figure 4D). Under conditions of enzyme excess, the catalytic rate was 0.0016 ± 0.0001 min^{-1} (Figure 5C), which is very similar to the value for k_{cat} obtained under multiple-turnover conditions.

The effects of temperature, pH, and Mg^{2+} concentration on the DNA-catalyzed reaction were explored for both the 2′:10-16 and L:15-30 DNA enzymes under single-turnover conditions. The optimal temperature for both enzymes was ~42 °C, which is slightly higher than that employed during the in vitro selection process (see Supporting Information). The optimal pH for the 2′:10-16 DNA enzyme was about 7.5, with reduced activity below pH 6.5 and above 8.5 (Figure 6A). The catalytic rate for the L:15-30 DNA enzyme was not dependent on pH over the range of 6.0−9.0 (Figure 6B), suggesting that the rate-determining step of the reaction is not the chemical step. The catalytic rates of both the 2′:10-16 and L:15-30 DNA enzymes were dependent on the concentration of Mg^{2+}, exhibiting saturation behavior in both cases. The apparent $K_d(Mg^{2+})$ for the 2′:10-16 enzyme was ~4 mM, while that for the L:15-30 enzyme was ~0.6 mM (see Supporting Information).

Several different divalent metal cations were tested for their ability to support catalysis by the 2′:10-16 and L:15-30 DNA enzymes. The 2′:10-16 DNA enzyme showed the highest level of activity in the presence of Mg^{2+}, with progressively lower activity in the presence of Ca^{2+}, Sr^{2+}, or Ba^{2+} and little or no activity in the presence of Mn^{2+}, Pb^{2+}, Cd^{2+}, Co^{2+}, or Zn^{2+}. The L:15-30 DNA enzyme was most active in the presence of Mn^{2+}, with progressively lower activity in the presence of Mg^{2+}, Ca^{2+}, or Pb^{2+} and little or no activity in the presence of Ba^{2+}, Sr^{2+}, Cd^{2+}, Co^{2+}, or Zn^{2+} (see Supporting Information). Neither enzyme exhibited activity in the presence of $Co(NH_3)_6$ (data not shown).

The cleavage products resulting from the reaction with both the 2′:10-16 and L:15-30 DNA enzymes were analyzed by high-resolution PAGE and MALDI mass spectrometry. In both cases the 5′-cleavage product was an oligonucleotide of the expected length, terminating in either a 2′,3′-cyclic phosphate or a 2′- or 3′-monophosphate (see Figure 7 and Supporting Information). The 3′-cleavage product also was of the expected length and terminated in a free 5′-hydroxyl, as confirmed by MALDI mass spectrometry (see Supporting Information).

The regio- and enantiospecificity of the 2′,5′-phosphodiester-cleaving and L-ribonucleotide-cleaving DNA enzymes, respectively, were determined under single-turnover conditions, comparing substrates that contained either the unnatural or a natural ribonucleotide at the cleavage site (compare Figures 3 and 8). The uncatalyzed rate of cleavage also was measured for the various substrates (see Supporting Information). The 2′:15-2 DNA enzyme exhibited a k_{cat}/k_{uncat} of ~20 000 for the substrate containing a 2′,5′-linked ribonucleotide and a k_{cat}/k_{uncat} of 3.3 for the corresponding substrate containing a 3′,5′-linked ribonucleotide, reflecting a regioselectivity of about 6000-fold in favor of the unnatural substrate. The 2′:10-16 DNA enzyme exhibited a regioselectivity of about 2000-fold. The L:15-30 DNA enzyme was less selective than the 2′,5′-cleaving DNA

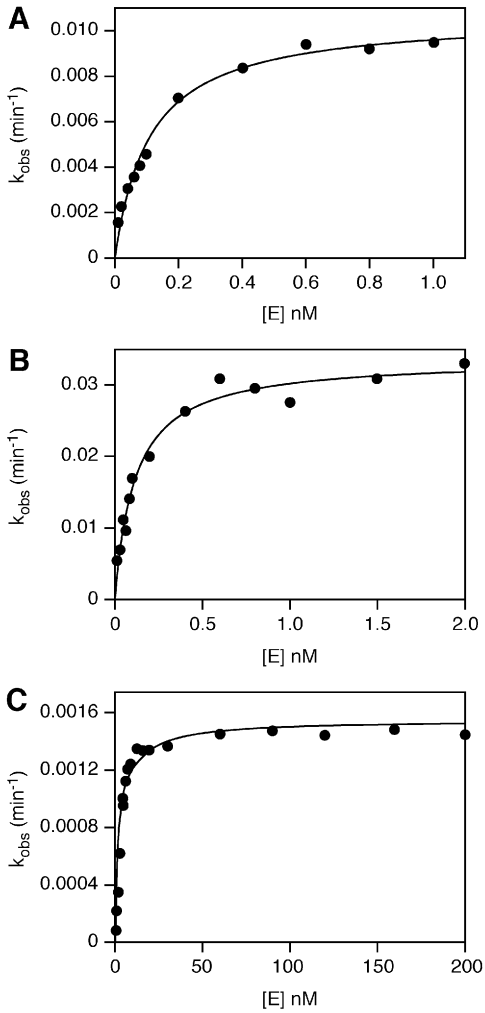

Figure 5. Catalytic activity of DNA enzymes that cleave unnatural ribonucleotide analogues, measured under single-turnover conditions. k_{obs} was determined for various concentrations of enzyme, and the data were fit to a curve based on the following equation: $k_{obs} = k_{max}[\text{enzyme}]/(K_d + [\text{enzyme}])$, where $k_{max} = k_{cat}$ at saturating concentrations and K_d is the apparent dissociation constant for the enzyme−substrate complex. Key: (A) 2′:10-16 DNA enzyme, with a k_{cat} of 0.011 ± 0.0004 min^{-1} and K_d of 0.11 ± 0.01 nM; (B) 2′:15-2 DNA enzyme, with a k_{cat} of 0.034 ± 0.001 min^{-1} and K_d of 0.12 ± 0.01 nM; (C) L:15-30 DNA enzyme, with a k_{cat} of 0.0016 ± 0.0001 min^{-1} and K_d of 3.2 ± 0.5 nM. Reaction conditions: 25 mM $MgCl_2$; 150 mM NaCl; pH 7.5; 37 °C.

enzymes. For the substrate containing an L-ribonucleotide, k_{cat}/k_{uncat} was ∼500, while, for the corresponding substrate containing a D-ribonucleotide, k_{cat}/k_{uncat} was ∼13. This corresponds to an enantioselectivity of about 40-fold in favor of the unnatural substrate. The regio- or enantioselectivity of the 2′,5′-phosphodiester- or L-ribonucleotide-cleaving DNA enzymes, respectively, is demonstrated in the autoradiogram shown in Figure 7.

Discussion

The substrate specificity of an enzyme is determined by its ability to discriminate both at the step of substrate binding and at the chemical step of the reaction. For small-molecule substrates it generally is more difficult to achieve a high degree of discrimination compared to macromolecular substrates because of the smaller number of potential interactions between the enzyme and small molecule. The interaction between two nucleic acid molecules can be highly specific, based on sequence recognition involving Watson−Crick base pairing as well as

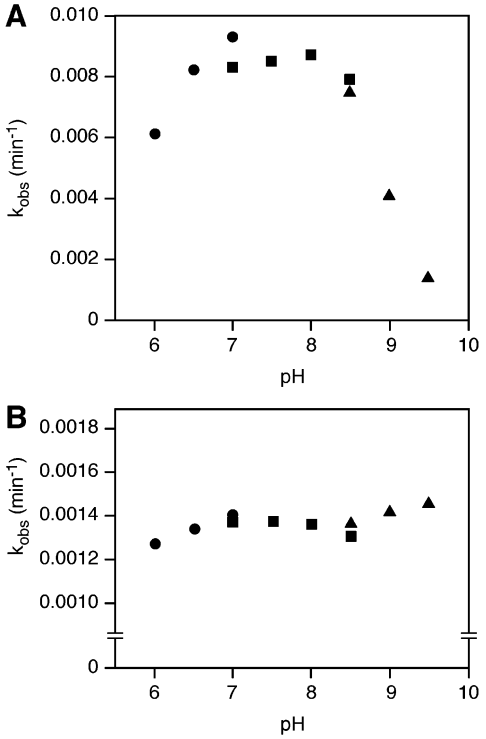

Figure 6. pH dependence of the DNA-catalyzed reactions: (A) 2′:10-16 DNA enzyme; (B) L:15-30 DNA enzyme. The buffer was either MES (●), EPPS (■), or CHES (▲).

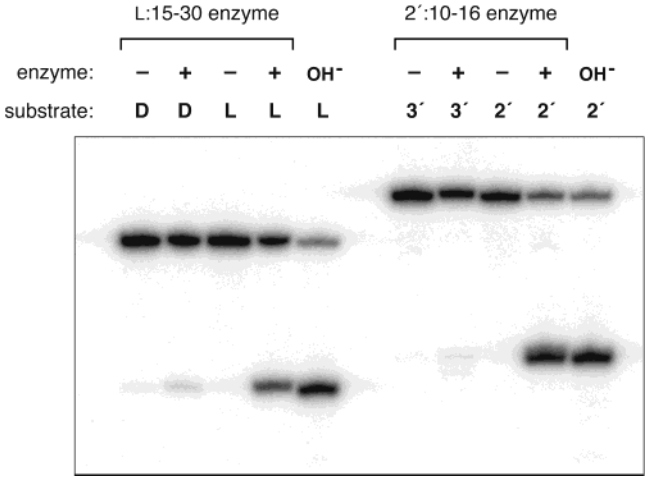

Figure 7. Autoradiogram depicting the cleavage reaction catalyzed by either the L:15-30 DNA enzyme or 2′:10-16 DNA enzyme, each with either its corresponding unnatural ribonucleotide substrate or a substrate in which the unnatural ribonucleotide was replaced by a standard ribonucleotide. Reaction conditions: 25 mM $MgCl_2$; 150 mM NaCl; pH 7.5; 37 °C; incubated in either the presence (+) or absence (−) of the DNA enzyme for 6 h. The unnatural ribonucleotide substrates also were subjected to alkaline hydrolysis (OH^-) by incubating them in the presence of 0.1 N NaOH for 6 h at 37 °C.

nonstandard pairing interactions. The ability of nucleic acid molecules to distinguish one another based on their regio- or enantioisomeric composition also has been explored. For example, 2′,5′-linked RNA is able to form stable duplexes with either 2′,5′- or 3′,5′-linked RNA but not with 3′,5′-linked DNA.[16] Similarly, 2′,5′-linked DNA can form stable duplexes with 3′,5′-

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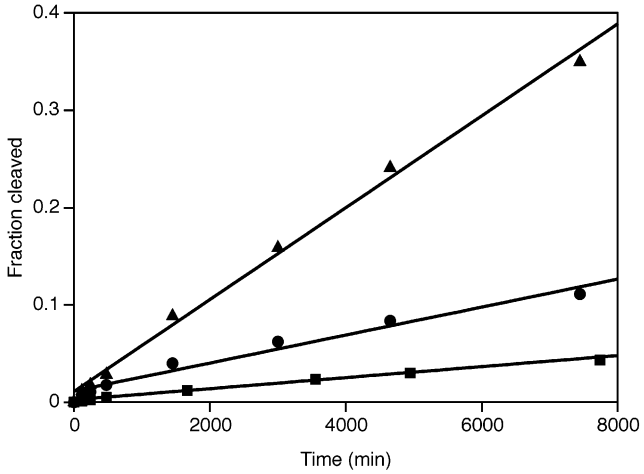

Figure 8. DNA-catalyzed cleavage of substrates that contained a natural ribonucleotide in place of the unnatural ribonucleotide. Reactions were carried out in the presence of saturating concentrations of DNA enzyme, employing either the 2′:10-16 DNA enzyme (●), 2′:15-2 DNA enzyme (■), or L:15-30 DNA enzyme (▲). The catalytic rate was obtained from a best-fit line of the data plotted as a function of time. Reaction conditions: 25 mM $MgCl_2$; 150 mM NaCl; pH 7.5; 37 °C.

linked RNA but not with 3′,5′-linked DNA.[17] An all-L-oligodeoxynucleotide composed of six adenylate residues was shown to pair with a complementary all-D-RNA strand but not with the corresponding all-D-DNA.[18] Another study, however, reported that all-L-DNA is unable to form duplexes with either all-D-RNA or all-D-DNA.[19] In the present study the substrates contained a single unnatural ribonucleotide, embedded within an otherwise all-natural DNA molecule, posing a more difficult challenge for either regio- or enantiospecific recognition.

The DNA enzymes that were obtained in this study are highly specific for substrates that contain a single unnatural ribonucleotide. One enzyme was able to distinguish between a 2′,5′- and 3′,5′-linked residue with a regiospecificity of 6000-fold. Another could distinguish between an L- and D- residue with an enantioselectivity of 40-fold. The catalytic rate of the 2′,5′-phosphodiester-cleaving DNA enzyme was ~0.01 min^{-1}, and that of the L-ribonucleotide-cleaving DNA enzyme was about 10-fold slower. These rates are significantly slower than the rate of other reported RNA-cleaving DNA enzymes that cleave natural ribonucleotides.[20−23] The "10-23" DNA enzyme, for example, can achieve a catalytic rate of up to 10 min^{-1} under optimal reaction conditions.[24] Perhaps the DNA enzymes that cleave unnatural ribonucleotides have difficulty folding into an active conformation or positioning a divalent metal cation to assist in the cleavage of the target phosphodiester. By analogy with known ribonucleases, the mechanism of cleavage likely involves deprotonation of the free 2′- or 3′-hydroxyl followed by attack of the resulting oxyanion on the adjacent phosphate. An in-line orientation is required for this attack, which can be achieved by forcing the nucleotide that precedes the cleavage site into an extrahelical position.[25] A single unpaired purine nucleotide within an otherwise complete duplex structure is especially amenable to achieving this orientation through local conformational changes, primarily involving the ϵ and ζ backbone torsion angles.[26] It may be more difficult for a 2′,5′-linked D-ribonucleotide, and especially a 3′,5′-linked L-ribonucleotide, to achieve the required orientation within the context of a Watson−Crick duplex. Thus, additional catalytic assistance may be required to bring about the cleavage of these unnatural ribonucleotides.

The uncatalyzed rate of cleavage of a 3′,5′-phosphodiester of RNA has been measured for both a single ribonucleotide embedded within an otherwise all-DNA molecule[27] and for an all-RNA oligomer.[28] The uncatalyzed rate of hydrolysis for the 2′,5′-phosphodiester of RNA, either in the presence or absence of a divalent metal cation, is similar to that of a 3′,5′-phosphodiester,[12,29−32] except when the RNA is bound to a complementary strand. In that case, the 2′,5′-linkage is about 7-fold more labile, whereas the 3′,5′-linkage is about 5-fold more stable.[33] To determine whether this "duplex effect" was partially responsible for the catalytic rate enhancement observed with the 2′,5′-cleaving DNA enzyme, the substrate containing the 2′,5′-linked ribonucleotide was hybridized to a complementary DNA strand and its hydrolysis rate in that context was compared to the hydrolysis rate of the substrate alone. No difference was seen in the uncatalyzed rate of cleavage under these two conditions (data not shown). The uncatalyzed rate of cleavage measured for a single embedded ribonucleotide was similar to that reported previously.[27,28]

The DNA enzymes developed in this study do not have any counterpart in nature and would not be able to function with any known biological substrate. They could, however, be useful as biochemical tools in cleaving a reporter molecule that contains an unnatural ribonucleotide. Such a reporter would not be cleaved by biological nucleases. One potential application of this activity pertains to a method for quantitative PCR, termed "DzyNA-PCR".[34] This method employs an RNA-cleaving DNA enzyme to cleave a reporter oligonucleotide that contains a fluorescent label and quencher on either side of the cleavage site. The sequence of the DNA enzyme is encoded by a complementary sequence that is attached to the 5′ end of one of the two PCR primers. As PCR amplification proceeds, functional copies of the DNA enzyme are produced. These can cleave the reporter molecule, separating the fluorescent label and quencher, and generating a fluorescent signal. Because the reporter contains natural ribonucleotides, it is susceptible to cleavage by biological ribonucleases. This would not be the case,

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however, if one employed a DNA enzyme that cleaves an unnatural ribonucleotide within the reporter molecule. There are naturally occurring ribonucleases that cleave 2′,5′-linked oligoadenylates[35]—these molecules are generated as part of the interferon response pathway. However, there is no known biological nuclease that cleaves either a 2′,5′-linked guanylate or an L-ribonucleotide.

In addition to their potential application as biochemical tools, the DNA enzymes described here illustrate that nucleic acid enzymes can exhibit substrate regio- and enantioselectivity comparable to that of their natural protein counterparts. Snake venom phosphodiesterase I, for example, can cleave either a D- or L-ribonucleotide, but is 1800-fold more active in cleaving the natural D-RNA substrate.[36] Further in vitro evolution experiments, especially those employing functionally enhanced nucleic acid analogues,[37,38] may lead to the development of novel catalysts with even greater regio- or enantioselectivity.

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Supporting Information Available: Sequences of individual clones isolated following the 15th round of in vitro selection, figures depicting k_{obs} as a function of Mg^{2+} concentration, temperature, and choice of divalent metal cation, and MALDI mass spectra of the products of the DNA-catalyzed reactions (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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