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Probing the Function of Nucleotides in the Catalytic Cores of the 8−17 and 10−23 DNAzymes by Abasic Nucleotide and C3 Spacer Substitutions†

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ABSTRACT: 8−17 and 10−23 are the two most comprehensively studied RNA-cleaving DNAzymes to date and have the ability to carry out sequence-specific cleavage of both all-RNA or chimeric RNA/DNA substrates. Mutagenesis studies of 8−17 and 10−23 DNAzymes using alternative natural nucleotides to substitute a given nucleotide in the DNAzyme sequence have found that both DNAzymes are able to tolerate a variety of alterations at many sequence locations. Chemical modification studies employing nucleotides containing nonnatural nucleobases have led to findings that some specific entities of selected nucleobases are irreplaceable by other functional groups. In this work, we set out to carry out a mutagenesis study on both 8−17 and 10−23 by substituting individual nucleotides in their catalytic cores with a baseless (abasic) nucleotide or a baseless/sugarless nucleotide containing only acyclic C3 spacer. We observed that the substitution with an abasic nucleotide or C3 spacer at many locations within the catalytic core of both 8−17 and 10−23 was still able to support a significant level of catalytic activity of each DNAzyme, suggesting that both DNAzymes have considerable structural plasticity to maintain their catalytic functions. We also observed that almost all nucleobases in the catalytic core of each DNAzyme appeared to make either an absolutely essential contribution to the function of each DNAzyme or exhibit a "chaperone-like" activity that is important for the optimal function of each DNAzyme; in contrast, only one sugar ring in 8−17 and four in 10−23 were inferred to make some contribution to the optimal function of the relevant DNAzyme. Finally, our study also raised a possibility that the 10−23 DNAzyme might be a special structural variant of the larger 8−17 DNAzyme family.

Catalytic DNAs (also known as deoxyribozymes or DNAzymes) are man-made single-stranded DNA molecules with the ability to catalyze chemical reactions. Since the description of the first DNAzyme for the cleavage of RNA in 1994 (*1*), many more DNAzymes have been reported (*2*, *3*) to catalyze many different types of chemical transformations, such as porphyrin metalation (*4*), DNA phosphorylation (*5*), RNA ligation (*6*), thymine−thymine dimer repair (*7*), carbon−carbon bond formation (*8*), and hydrolytic cleavage of DNA (*9*). There are several reasons to investigate the catalytic capability of DNA. From an academic point of view, studying simple DNAzymes could reveal important information that could deepen our understanding of basic principles that govern enzymatic catalysis. From an application point of view, developing DNAzyme-based tools offers certain advantages. DNA is chemically stable and can be conveniently produced by highly efficient automated DNA synthesis. Therefore, DNAzymes can be quite useful in research and applications in chemical biology, biotechnology, and medical areas.

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RNA-cleaving DNAzymes are the best studied catalytic DNA molecules currently available, given their potential as therapeutic agents and diverse molecular tools (*3*, *10*−*12*). To date, several classes of RNA-cleaving DNAzymes have been identified from random-sequence DNA pools by in vitro selection (*12*). These motifs are characterized by distinct primary sequences and secondary structures, unique cofactor and pH dependencies, and specific substrate requirements (*13*−*21*).

8−17 and 10−23 are the two most comprehensively studied RNA-cleaving DNAzymes and have the ability to carry out sequence-specific cleavage of all-RNA or chimeric RNA/DNA substrates (*19*). 8−17 has been examined for several potential applications (*22*), including nucleic acid detection (*23*, *24*), metal ion sensing (*25*), and DNA computing (*26*). 10−23, on the other hand, has been quite widely examined as a potential therapeutic agent to suppress the level of specific RNAs in cells (*27*−*35*).

Both 8−17 and 10−23 are small in size and composed of a catalytic domain flanked by two substrate-binding arms (see their secondary structures illustrated in Figure 1). The catalytic domain of 8−17 has ∼15 nucleotides composed of a three-base-pair stem, an AGC triloop, and four to five unpaired nucleotides. Similarly, the catalytic core of 10−23 is also made of ∼15 nucleotides; however, no base-pairing elements have been defined for its catalytic core.

8−17 and 10−23 have also been subjected to detailed investigations aimed to define their sequence requirements, global

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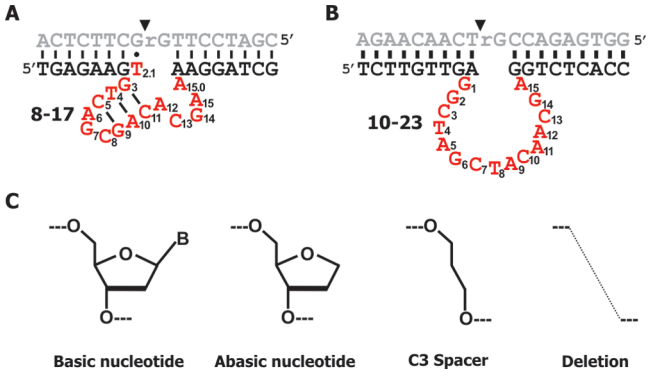

FIGURE 1: (A) The secondary structure of 8−17. The nucleotide positions within the catalytic core of 8−17 are numbered according to Peracchi et al. (*43*). (B) The secondary structure of 10−23. Nucleotides in the catalytic core are numbered according Zaborowska et al (*45*). The substrate (gray) for each DNAzyme is a chimeric DNA/RNA molecule containing a ribonucleotide (guanosine nucleotide, denoted rG) as the cleavage site. (C) Schematic drawings of standard nucleotide, abasic nucleotide, C3 spacer, and nucleotide deletion used in the upcoming figures.

folding, and catalytic mechanisms (*20*, *22*, *36*−*43*). For example, FRET[1] (fluorescence resonance energy transfer) analyses conducted to examine the folding of 8−17 (*36*, *39*−*41*) suggest that in the presence of Pb^{2+} 8−17 intrinsically folds into a prearranged, catalysis-ready conformation; however, in the presence of other metal ions (such as Zn^{2+} and Mg^{2+}), the same DNAzyme experiences a conformational change to carry out its catalysis. Conformational change preceding the cleavage reaction has also been revealed by tracking the flow of electron holes from one end of the substrate binding helices in the presence of Zn^{2+} or Mg^{2+} (*44*).

Mutagenesis is a technique widely used to probe the significance and dispensability of a particular nucleotide in a functional nucleic acid molecule; this approach has been applied to examine the function of both the 8−17 and 10−23 DNAzymes. For example, several mutagenesis studies (*15*, *19*, *37*, *43*) have revealed the following four absolutely conserved nucleotides in the catalytic core of 8−17: A_6, G_7, C_{13}, and G_{14} (Figure 1A) (*22*). Mutagenesis studies have also been conducted for 10−23 (*45*, *46*), which led to the suggestion that the catalytic core of 10−23 may be composed of eight nucleotides at the two borders, specifically G_1, G_2, C_3, T_4, A_5, G_6, C_{13}, and G_{14} (Figure 1B).

Standard DNAzyme mutagenesis typically involves nucleotide deletion or the use of alternative natural nucleotides to substitute a given nucleotide in a DNAzyme sequence, followed by the activity analysis of the resultant mutants. Since such manipulations can potentially alter molecular interactions (such as hydrogen bond network, base stacking, steric repulsion, and metal ion stabilization) crucial to the function of a DNAzyme, this simple and convenient approach can reveal valuable information about the contribution of the concerned nucleotide in the catalytic function of the DNAzyme. Substitution of a nucleotide in a DNAzyme with unnatural nucleotide analogues can also be a highly valuable approach in probing the roles of individual functional groups on the concerned nucleotide. For example, using nucleotide analogues containing nonnatural nucleobases to substitute the conserved nucleotides in 8−17 has identified the N7 atom of adenine at A_6, the 6-keto group of guanine at G_7, and the N1 imino and 2-amino groups of guanine at G_{14} to be some of the most crucial functional groups that confer the catalytic activity of 8−17 (*43*).

In this work, we set out to carry out a mutagenesis study on both 8−17 and 10−23 using a novel approach of substituting individual nucleotides in their catalytic cores with a baseless (abasic) nucleotide or a baseless/sugarless nucleotide containing only C3 spacer (i.e., $CH_2CH_2CH_2$) (see Figure 1C). Such an approach can have two advantages. First, replacing a given nucleotide with an abasic nucleotide or a C3 spacer makes it possible to define the contribution (or lack of it) of the nucleobase and sugar ring of a nucleotide of interest without interference from undesirable new interactions that might be created by the use of a different base-containing nucleotide. Second, a "chaperone-like" function (with possible chemical interactions that contribute to the tertiary structural formation of the DNAzyme) or a passive spacer function (without contributing chemical interactions) of a nucleobase may be inferred by this approach.

MATERIALS AND METHODS

Preparation of Oligonucleotides and Chemical Reagents. Standard and modified oligonucleotides were either prepared by solid-phase synthesis using cyanoethyl phosphoramidite chemistry or purchased from Keck Biotechnology Resource Laboratory, Yale University. Deoxyribonucleotide and ribo-G phosphoramidites were purchased from GenePharma (Shanghai, China). Abasic and C3 spacer phosphoramidites were synthesized from 2′-deoxyribose and 1,3-propanediol, respectively, according to the published protocols (*47*, *48*). Chemically synthesized phosphoramidites were structurally validated by ^{1}H NMR and ^{31}P NMR before use. All oligonucleotides were purified by preparative denaturing (8 M urea) 8% polyacrylamide gel electrophoresis (PAGE), and their concentrations were determined spectroscopically at 260 nm. Chemical reagents were purchased from Sigma.

The protecting TOM (triisopropylsilyloxymethyl) group or TBDMS (*tert*-butyldimethylsilyl) group of the RNA linkage within the chimeric DNA/RNA substrates was removed by adding 500 μL of TBAF (tetrabutylammonium fluoride; 1 M solution in tetrahydrofuran; Sigma) to the dry pellet of substrate (~250 pmol), followed by overnight incubation at 60 °C. The reaction was quenched by adding 200 μL of H_2O. The resulting product was recovered by ethanol precipitation, desalted on a Nanosep 1K filter (Pall Life Sciences), and purified by 8% denaturing PAGE. Deprotected substrate was radiolabeled at the 5′ end with 10 μCi/μL [γ-^{32}P]ATP (Furui, China) at 37 °C for 60 min with T4 polynucleotide kinase (Takara Biotech, Japan). Radiolabeled substrate was purified by 8% denaturing PAGE.

Kinetic Analyses. All cleavage reactions were conducted under single turnover conditions, in which each 8−17 or 10−23 DNAzyme was at 200- and 100-fold in excess over the corresponding substrate ([substrate] = 5 nM). For 8−17, the reaction was performed in 50 mM HEPES (pH 7.5), 200 mM NaCl, and 10 mM $MgCl_2$ at 37 °C. For 10−23, the reaction was performed in 50 mM HEPES (pH 8.0), 200 mM NaCl, and 500 mM $MgCl_2$. The substrate and the DNAzyme were separately preincubated in the reaction buffer at 37 °C for 10 min before mixing to initiate

[1]Abbreviations: FRET, fluorescence resonance energy transfer; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; TOM, triisopropylsilyloxymethyl; TBDMS, *tert*-butyldimethylsilyl; TBAF, tetrabutylammonium fluoride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; NaOAc, sodium acetate; WT, wild type.

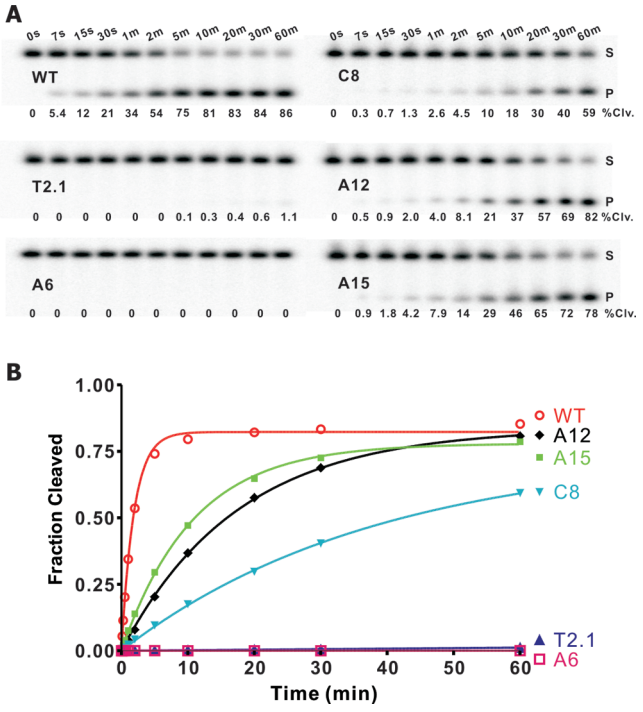

FIGURE 2: (A) PAGE-based assays and (B) kinetic plots of the wild-type 8−17 DNAzyme (WT) and five representative 8−17 mutants with abasic substitution at $T_{2.1}$, A_6, C_8, A_{12}, and A_{15}. All cleavage reactions were conducted under single turnover conditions (DNAzyme:substrate = 200:1) for the specified time points. S: uncleaved substrate labeled with ^{32}P at 5′ end; P: 5′-cleavage fragment. Three independent experiments were conducted for each construct, one of which is shown in the figure. The kinetic data were fitted according to the one-phase exponential equation $Y = Y_m(1 - e^{-kt})$: Y, cleavage yield; t, reaction time; Y_m, maximal cleavage yield; k, observed rate constant (k_{obs}). The Y_m and k_{obs} values of these mutants are provided in Table S1.

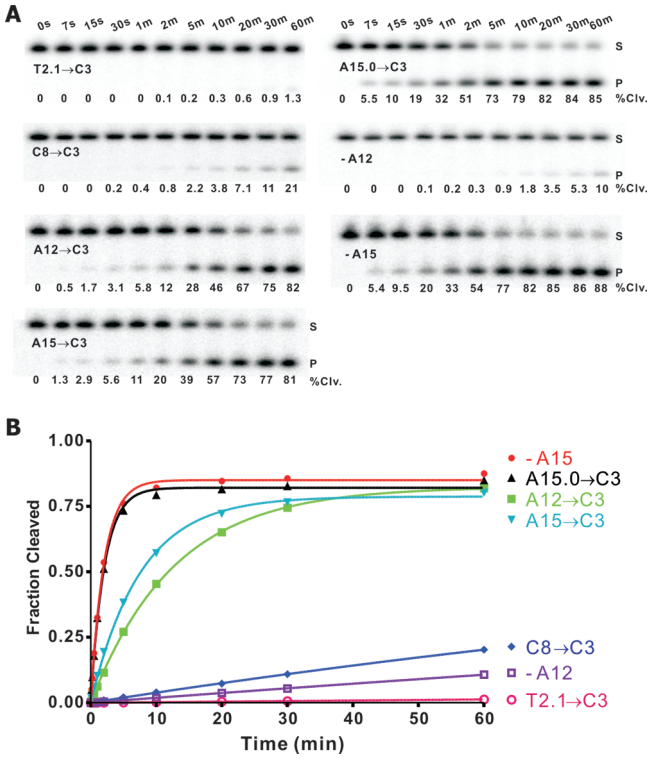

FIGURE 3: (A) PAGE-based assays and (B) kinetic plots of 8−17 mutants with C3 substitution at $T_{2.1}$, C_8, A_{12}, A_{15}, $A_{15.0}$, and with deleted A_{12} and A_{15}. Experimental design and data analyses were conducted similarly as described in Figure 2 legend.

the cleavage reaction. An aliquot of 50 μL was withdrawn from the reaction mixture at 7, 15, 30 s, 1, 2, 5, 10, 20, 30, and 60 min (for 8−17) or 7, 15, 30 s, 1, 2, 5, 10, 30, 60, 90, and 120 min (for 10−23) and immediately mixed with 2 μL of 0.5 M EDTA (pH 8.0). This was followed by the addition of NaOAc (pH 7.0) to a final concentration of 300 mM and 2.5× equivalent volume of ice-cold ethanol. The DNA molecules in the mixture were precipitated by centrifugation and analyzed by 10% denaturing PAGE. Phosphorimages of the PAGE-resolved reaction mixtures were obtained by using a Molecular Dynamics Storage Phosphor Screen and a GE Biosciences Typhoon Trio Imager. Cleavage fractions were quantified by using the ImageQuant Software. Three independent experiments were performed with each DNAzyme construct, and the data obtained were fitted according to one-phase exponential equation $Y = Y_m(1 - e^{-kt})$, using GraphPad Prism 4.03 where Y is the cleavage yield (in percent) at a given reaction time, to obtain maximal cleavage yield Y_m and observed rate constant k (denoted k_{obs} in the remainder of the report). For some weak mutants, a 24 h time point was also taken to verify the extrapolated Y_m (see more information provided in Results section below).

RESULTS

Nucleotide Substitutions in the Catalytic Core of 8−17. The unsubstituted 8−17 DNAzyme used in this study (denoted 8−17WT) contains 15 nucleotides in its catalytic core (Figure 1A). We made the abasic substitution at each unpaired nucleotide (nine in total) but did not make such replacement for the six nucleotides in the well-defined three-base-pair stem. The cleavage assay for 8−17WT and each mutant was conducted under single turnover conditions with the use of a chimeric DNA/RNA substrate labeled with ^{32}P at its 5′ end (its sequence is shown in gray in Figure 1A). Reaction mixtures taken at specific time points were analyzed by denaturing PAGE to resolve the radioactive 5′-cleavage fragment from the uncleaved substrate. PAGE-based results obtained with 8−17WT and abasic substitution at $T_{2.1}$, A_6, C_8, A_{12}, and A_{15} are provided in Figure 2A. The percent cleavage at various time points was calculated, and the data were then curve-fitted to derive observed rate constant (k_{obs}) and the maximal cleavage yield (Y_m) for each DNAzyme construct; kinetic plots for these DNAzyme constructs are shown in Figure 2B. PAGE-based results for abasic substitutions at G_7, C_{13}, G_{14}, and $A_{15.0}$ are provided in Figure S1A in Supporting Information; the corresponding kinetic plots are illustrated in Figure S1B (note: the kinetic data for A15.0 in particular is very similar to that of 8−17WT).

Nucleotides that showed detectable activity upon abasic replacement were then subjected to C3 spacer substitution. Such modification was made for $T_{2.1}$, C_8, A_{12}, A_{15}, and $A_{15.0}$; PAGE-based results of these five constructs are provided in Figure 3A, and kinetic plots are shown in Figure 3B. If the C3 spacer substitution did not significantly reduce the DNAzyme activity, a mutant was then examined where the entire nucleotide was deleted. Such manipulation was carried out only for A_{12} and A_{15}; PAGE-based results and kinetic profiles are also provided in panels A and B of Figure 3, respectively.

The k_{obs} and Y_m values for all of the 8−17 variants examined are listed in Table S1. For the convenience of comparison, we chose to normalize the k_{obs} and Y_m values of each mutant DNAzyme

against those of 8−17WT (which were taken as 100) using the equation of 100[(k_{obs} or Y_m)$_{mutant}$/(k_{obs} or Y_m)$_{WT}$]. The calculated relative k_{obs} and Y_m values are shown in Table S2, which are also organized into a summary figure, Figure 4, to facilitate the discussion below.

It should be noted that for some of the weakly active mutants of 8−17 (and 10−23 to be discussed later below) a 24 h reaction was conducted to verify the accuracy of the extrapolated Y_m. The data for these mutants are provided in Figure S5. The extrapolated Y_m and the observed Y_{24} are consistent for all of the mutants, with variations typically smaller than 15%.

Substitution at $T_{2.1}$. Substitution with either an abasic nucleotide (Figure 2) or a C3 spacer (Figure 3) at position 2.1 is significantly detrimental to the activity of 8−17: ~1% substrate cleavage was observed in 60 min (Figure 2A); even when the reaction time was extended to 24 h, only 23% cleavage was seen (Figure S5A). For comparison, 8−17WT produced 81% substrate cleavage in 10 min (Figure 2A; also see Figure 4A for the relative k_{obs} and Y_m data).

Substitutions at the $A_6G_7C_8$ Triloop. Replacement of either A_6 (Figure 2A) or G_7 (Figure S1A) with abasic nucleotide completely deactivated 8−17. Mutation of C_8 to the abasic nucleotide significantly reduced the catalytic activity (Figure 2A). Kinetic analysis (Figure 2B) indicated that k_{obs} of the construct was severely affected (relative k_{obs} decreased to 5 on the scale of 100 in reference to 8−17WT; Figure 4B); however, Y_m was not reduced (Figure 4B; Figure S5A). The mutation of C_8 to C3 spacer further reduced the k_{obs} by 5-fold but again did not affect Y_m (Figure 4B; Figure S5A).

Substitutions at the $A_{12}C_{13}G_{14}A_{15}A_{15.0}$ Pentaloop (Figure 4C). The abasic or C3 substitution at A_{12} did not affect Y_m but reduced k_{obs} considerably (11 and 15, respectively). Deletion of this nucleotide, however, had a potent impact on k_{obs} (to only 1) but only a small effect on Y_m. Abasic substitution for C_{13} or G_{14} rendered 8−17 completely inactive. The 8−17 mutants with the abasic or C3 substitution at A_{15} behaved similarly to the mutants with the corresponding replacements at A_{12}: little impact on Y_m but considerable reduction to k_{obs}. Interestingly, deletion of A_{15} had little effect on both k_{obs} and Y_m. Abasic and C3 substitution at $A_{15.0}$ produced minimal effect on the catalytic activity as these constructs exhibited k_{obs} and Y_m values that were similar to that of 8−17WT. However, when both A_{15} and $A_{15.0}$ were deleted, k_{obs} dropped by ~25-fold whereas Y_m was not affected.

Nucleotide Substitutions in the Catalytic Core of 10−23. The catalytic core of the unsubstituted 10−23 DNAzyme used in this study (10−23WT) also contains 15 nucleotides (Figure 1B), all of which were subjected to abasic substitution. Similar to the 8−17 studies above, the cleavage assay for the wild type and each mutant was conducted under single turnover conditions with the use of a chimeric DNA/RNA substrate labeled with ^{32}P at its 5′ end (its sequence is shown in gray in Figure 1B). The PAGE-based results obtained for 10−23WT and G_1−C_7 mutants are shown in Figure S2; those for T_8−A_{15} mutants are provided in Figure S3. Substitution with C3 spacer was performed for G_1, G_2, C_3, T_4, C_7, T_8, A_9, C_{10}, A_{11}, A_{12}, and A_{15}; the PAGE-based results are shown in Figure S4. One deletion construct, the T_8 detection mutant, was tested; the PAGE-based result is also provided in Figure S4.

Kinetic plots of 10−23WT and all of the mutants mentioned above are provided in Figure 5, with which the corresponding k_{obs} and Y_m values were derived and are listed in Table S3. Once again, for easy comparison of DNAzyme activities, both the k_{obs} and Y_m of 10−23WT are treated as 100 so that we can normalize the k_{obs} and Y_m values for other 10−23 variants. The data are provided in Table S4 and reorganized into a summary figure, Figure 6.

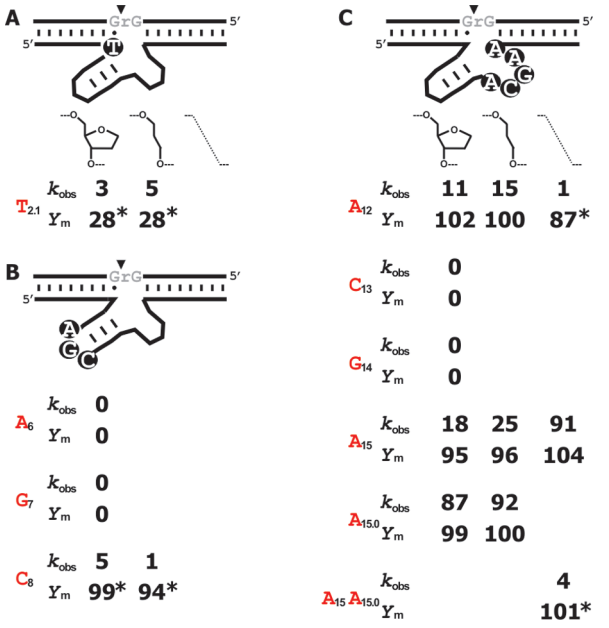

FIGURE 4: Relative k_{obs} and Y_m values for 8−17 variants examined in this study. The observed rate constant (k_{obs}) and maximal cleavage yield (Y_m) of the wild-type 8−17 are both taken as 100. The relative k_{obs} for a given mutant is calculated as 100[(k_{obs})$_{mutant}$/(k_{obs})$_{wild\ type}$]. Similarly, relative Y_m for a given mutant is calculated as 100[(Y_m)$_{mutant}$/(Y_m)$_{wild\ type}$]. Each nucleotide in the catalytic core (except those in the helical region) was replaced with an abasic nucleotide; those that retained significant level of activity were then subjected to C3 spacer replacement, followed by the deletion of the entire nucleotide if the significant activity was again observed. The results are grouped into panels A, B, and C for easy viewing in relation to the location of a concerned nucleotide in the catalytic core. An asterisk denotes that the relative Y_m was calculated with the observed yield from the 24 h reaction.

Substitutions at A_5, G_6, C_{13}, and G_{14} (Figure 6A). Abasic nucleotide replacements at these four positions completely or near completely abolished the cleavage activity.

Substitutions at G_2, T_4, C_7, A_9, C_{10}, A_{11}, A_{12}, and A_{15} (Figure 6B). Substitution of each of these nucleotides with the abasic nucleotide caused very substantial reduction of k_{obs} but no or less significant change to Y_m: All the mutants exhibited a k_{obs} value that is ~6−25-fold smaller than that of 10−23WT and yet maintained a high level of Y_m (~50−100% of Y_m of the 10−23WT). For example, the relative k_{obs} values of the three mutants with the abasic substitution at G_2, A_{11}, or A_{12} were reduced from 100 to 15, 9, and 12, respectively; in contrast, their Y_m values were essentially unchanged.

Mutating each nucleotide at these locations into C3 spacer did not alter k_{obs} significantly further beyond the abasic substitution. For Y_m, no or small changes (in comparison to those of abasic mutants) were observed: although the C3 spacer mutation at T_4, A_9, C_{10}, A_{11}, A_{12}, and A_{15} exhibited Y_m essentially identical to that of each corresponding abasic replacement, similar substitution at G_2 and C_7 showed a small reduction in Y_m. However, these small changes could simply reflect experimental errors.

Substitutions at G_1, C_3, and T_8 (Figure 6C). The abasic substitution at both G_1 and C_3 reduced k_{obs} by a detectable

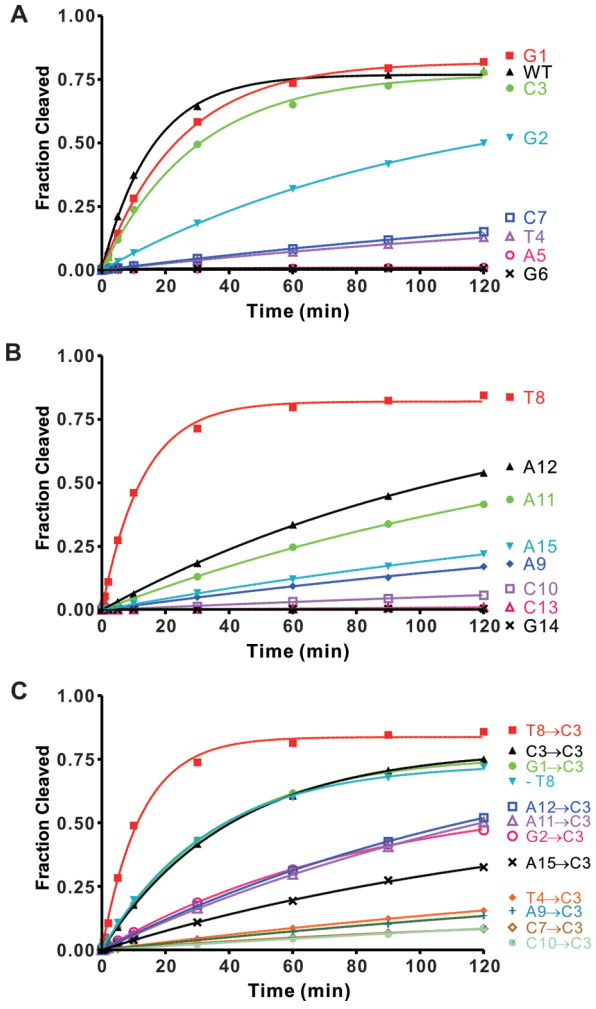

FIGURE 5: The kinetic plots of 10−23WT and all of the 10−23 variants examined in this study. Experimental design and data analyses were conducted similarly as described in Figure 2 legend.

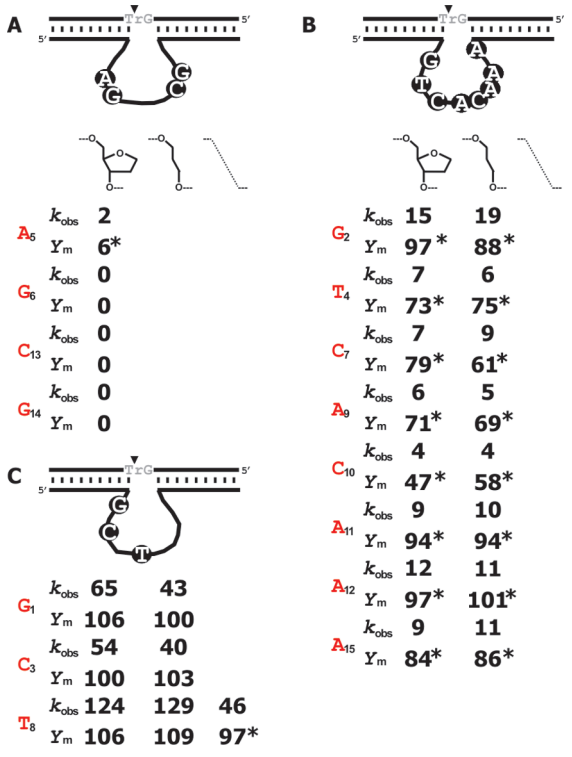

FIGURE 6: Relative k_{obs} and Y_m values for 10−23 variants examined in this study. Refer to the legend of Figure 4 for calculating relative k_{obs} and Y_m for a given mutant of each nucleotide.

degree (to 65 and 54, respectively) but did not affect Y_m. Replacement with the C3 spacer at each location produced a small further reduction in k_{obs} (to ~40) but again had little impact on Y_m.

Interestingly, the mutants with the removal of nitrogenous base (abasic substitution) and pentose ring (C3 spacer replacement) of T_8 exhibited slightly increased k_{obs} (124 and 129, respectively) and essentially unchanged Y_m (106 and 109). However, when T_8 was deleted entirely, k_{obs} had 54% decrease while Y_m was maintained close to the wild-type level.

DISCUSSION

To probe the function of 8−17 and 10−23, we made a variety of mutants by substituting each nucleotide in the catalytic core of the two DNAzymes and compared their activities to the wild-type sequences. The results obtained using this approach have not only validated many previous findings about essentiality and dispensability of individual nucleotides in the catalytic core of both DNAzymes but also revealed some new information that can better define the functional roles (or lack of them) of both nucleobase and sugar components on these nucleotides. In this section, we will make functional interpretation of our experimental observations in the context of the findings from previous mutagenesis studies.

Roles of Bases and Pentose Rings within the Catalytic Core of 8−17. *$T_{2.1}$.* Previous studies have shown that $T_{2.1}$ → C, A, or G mutations are detrimental to the DNAzyme activity when the cleavage site is a rN-G junction (N = A, C, G, and U) (*18*, *43*). It is now generally accepted that the base on $T_{2.1}$ forms a functionally important G·T wobble pair with the G residue at the cleavage junction of the substrate. It should be noted that our group previously reported that some 8−17 variants are capable of cleaving many other dinucleotide junctions, and more importantly, for those variants the absence of $T_{2.1}$ is necessary, along with appropriate mutations at other positions in the catalytic core (*15*, *37*). In other words, $T_{2.1}$ can be beneficial or detrimental to the function of 8−17, depending on the nature of dinucleotide junction to be targeted: this nucleotide plays a crucial role for 8−17 for the cleavage of rN-G junctions but is detrimental to 8−17 when the other dinucleotide junctions are the cleavage sites (*15*, *18*, *37*). As expected, we found that the abasic substitution at $T_{2.1}$ significantly reduced the DNAzyme activity. The pentose ring at this location (depicted as gray "s" in Figure 7A) does not appear to make any significant contribution to the DNAzyme activity based on our observation that the abasic and C3 spacer mutants exhibited almost identical k_{obs} and Y_m.

A_6, G_7, C_{13}, and G_{14}. Previous mutagenesis studies have revealed that the nucleobases on these four nucleotides are absolutely conserved, implying their crucial roles in the function of 8−17 (*15*, *37*, *43*). These nucleobases most probably make essential contributions to the formation of the catalytic core and may even participate directly in the chemical transformation process. Abasic nucleotide substitutions at these locations are expected to abolish the functional contributions of these nucleobases and should render the DNAzyme totally inactive, which was observed in this study. The sugar rings (black "s" letters in

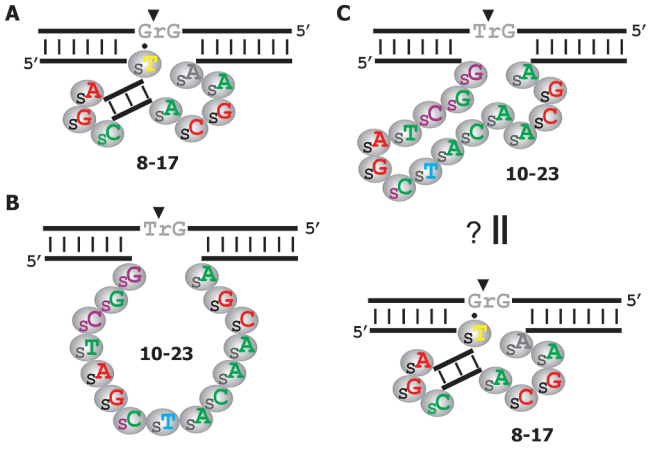

FIGURE 7: (A, B) Mapping of contribution of the base (letter in larger font in each circle) and pentose ring (letter "s" in each circle) of each concerned nucleotide to the function of the 8−17 (A) and 10−23 (B) DNAzyme. Capital letters A, C, G, and T, nucleobases; lower-case letter s, pentose rings. Color scheme: red, nucleobases that are crucial to the function of each DNAzyme; green (or purple), nucleobases or pentose rings that may make substantial (or small, if any) contribution to the optimal function of each DNAzyme; gray, nucleobases or pentose rings that do not appear to make any contribution to the function of each DNAzyme; black, pentose rings that were not examined. The yellow and blue T: the two special thymine bases as discussed in the main text. (C) Arrangement of nucleotides in the catalytic cores of 10−23 and 8−17 to provide a visual support for the proposal that 10−23 might be a special variant of 8−17.

Figure 7A) were not subjected to the C3 spacer substitution simply because the abasic mutants were already completely inactive.

C_8. Previous mutagenesis studies have shown that the cytosine base can be mutated to adenine, guanine, and thymine with substantial activity reduction (*15*, *37*, *43*). We found that the removal of this base from the nucleotide unit very significantly affected the rate of the cleavage reaction (by ~20-fold) but did not substantially reduce the maximal cleavage yield. Taken together, it appears that the cytosine base at this location (highlighted in green in Figure 7A) is important only to the *optimal* function of the DNAzyme. We term such a role as "chaperone-like" activity as we believe the cytosine does not participate directly in the chemical action but facilitates the most favorable positioning of the catalytic core. Because the C3 spacer mutant exhibited a further reduced k_{obs} (by 5-fold) while maintaining the identical Y_m in comparison to the abasic mutant (Figure 4B), the pentose ring at C_8 (green "s" in Figure 7A) appears to make some contribution to the proposed chaperone activity.

A_{12}. Previous mutagenesis studies have shown that substituting the adenine base at A_{12} with guanine, cytosine, and thymine does not significantly impact the catalytic activity (*15*, *37*, *43*). Our abasic substitution and nucleotide deletion data (Figure 4C) seem to suggest that the adenine base here may play two roles for the function of 8−17. The first is to act as an important physical spacer between the three-bp stem and the invariable nucleotide C_{13} so that C_{13} can be situated most adequately to carry out its catalytic function; therefore, deletion of A_{12} (which may alter functionally important molecular interactions, such as hydrogen bond network, base stacking, steric repulsions, metal ion stabilization, and torsion angles) would significantly change the physical arrangement of C_{13} in the catalytic core and result in a severe loss of activity. The second role is to act as a chaperone-like element that is important for the correct folding of the DNAzyme, given that the abasic substitution significantly decreased the rate of the DNAzyme-mediated reaction by ~9-fold but produced no impact to the maximal yield (Figure 4C). It appears that the adenine base, not the sugar ring, is the sole player for this role because both the abasic and C3 spacer substitutions had similar effects (Figure 4C).

A_{15} and $A_{15.0}$. Previous mutagenesis studies have shown that nucleobase mutations at these two locations can be well tolerated (*15*, *37*, *43*). In this study, abasic or C3 substitution at A_{15} did not affect Y_m but caused considerable reduction to k_{obs} (by ~5-fold; Figure 4C). A likely explanation is that the adenine base is a chaperone-like element that is important for the folding of the catalytic core into the active conformation. With the removal of this base, this chaperone-like function is lost, which would significantly affect the rate but not the maximal yield of the reaction, as observed. Once again, the sugar ring on this nucleotide appears to have little effect on the proposed chaperone-like activity because both the abasic and C3 spacer substitutions had almost identical outcomes. Interestingly, however, complete removal of A_{15} had little effect on both k_{obs} and Y_m, which paints a seemly perplexing role by A_{15}: the DNAzyme can produce an equally productive structure with and without this nucleotide.

This puzzling observation may be due to the presence of $A_{15.0}$, the neighboring nucleotide to the 3′-end of A_{15}: the adenine base on $A_{15.0}$ might have fulfilled the needed chaperone-like function when A_{15} is absent. In other words, $A_{15.0}$ itself in 8−17WT is neither beneficial nor detrimental to the function of 8−17 because the mutants with either abasic or C3 spacer substitution maintained wild-type-like activity (Figure 4C); however, it becomes beneficial and fulfills the needed chaperone-like role when the nucleotide A_{15} is removed. As discussed above, previous in vitro selection experiments have generated 8−17 variants with either an ACGA tetraloop or an ACGAA pentaloop (*15*, *19*, *21*), indicating that both the tetraloop and pentaloop are equivalent to the function of 8−17. Our result with abasic substitution at A_{15} indicates that there is a need for an adenine base on the nucleotide next to the highly conserved $C_{13}G_{14}$ motif for the optimal function of the DNAzyme. Once again, the sugar ring does not appear to make any contribution to the optimal activity (Figure 4C).

Further support for the needed chaperone-like activity by the adenine base at A_{15} came from the double deletion mutant when both A_{15} and $A_{15.0}$ were deleted: the k_{obs} of this mutant was significantly affected (by 25-fold) whereas Y_m was not (Figure 4C).

Roles of Bases and Pentose Rings within the Catalytic Core of 10−23. A_5, G_6, C_{13}, and G_{14}. The loss of activity in the mutants with abasic substitution at these four positions (Figure 6A) indicates that nucleobases on these nucleotides are vital to the function of 10−23. A previous study has found that the 6-keto group of G_6 and 4-amino group of C_{13} as well as the 2-amino group and 6-keto group of G_{14} are key to the catalytic function of 10−23 (*45*). Thus, it is not surprising that abasic substitution at these sites was detrimental to the cleavage activity. The catalytic contribution of A_5 is unclear from all the published studies conducted to date. Our finding that the activity of 10−23 was reduced dramatically when the adenine base on this nucleotide was removed (Figure 6A) and the previous finding that this same adenine can be substituted with cytosine (*45*) seem to suggest that the exocyclic amine group and the adjacent nitrogen

atom may engage themselves in some important interactions with other nucleotides in the catalytic core. The four sugar rings (black "s" letters) of these nucleotides were not subjected to C3 spacer substitution simply because each relevant abasic mutant was completely inactive or showed an extremely weak activity.

G_2, T_4, C_7, A_9, C_{10}, A_{11}, A_{12}, and A_{15}. The significant reductions in k_{obs} (by ~6−25-fold; Figure 6B) but no or less significant changes in Y_m in all abasic mutants made at these locations (Figure 6B) suggest that the base components of these nucleotides participate in interactions that are important to the optimal function of 10−23. These nucleobases most probably do not contribute directly to the catalytic chemistry but likely facilitate the placement of catalytically essential nucleotides in the proper setting to achieve optimal enzymatic activity. Based on the previous mutagenesis study where each nucleotide in the catalytic core was replaced with every other natural nucleotide, only G_2 and T_4 were found to be irreplaceable while the rest can be mutated to a different nucleotide (*45*). Thus, the nucleobases on G_2 and T_4 appear to have chaperone-like activity by contributing specific molecular interactions that facilitate proper tertiary structural formation of the DNAzyme. In contrast, position 7 can accept A, C, or T, positions 9 and 15 can take A, G, or T, while positions 10, 11, and 12 can tolerate any of the four nucleotides (*45*). Nucleobase promiscuity at these positions seems to suggest that these nucleotides merely act as a physical spacer and that the nucleobases do not contribute specific molecular interactions to the folding or catalysis of the DNAzyme.

All of the C3 spacer mutants at these locations exhibit almost identical k_{obs} and Y_m values seen with corresponding abasic mutants. However, the C3 spacer mutants at G_2 and C_7 produced a slightly reduced Y_m, suggesting that the pentose ring at these two locations (purple "s" letters) may make, at best, a very subtle positive contribution to function of the DNAzyme. However, the relative small differences (9% for G_2 and 18% for C_7) could simply reflect experimental inaccuracy.

G_1, C_3, and T_8. The observation that abasic mutants at G_1 and C_3 produced a small reduction in k_{obs} (< 2-fold) but no change in Y_m when compared to 10−23WT (Figure 6C) suggests that the bases at these two locations make, at best, a very subtle positive contribution to the optimal function of the DNAzyme. C3 spacer substitution at each location reduced k_{obs} slightly further but did not affect Y_m (Figure 6C), implying that the pentose rings on these two nucleotides make small, if any, contribution to the optimal function of 10−23. In other words, G_1 and C_3, under the best scenario, may act like weak chaperone-like elements to facilitate the proper positioning of the catalytic core.

Both the abasic and C3 spacer substitutions at T_8 produced a slightly better k_{obs} (~125 vs 100 for 10−23WT); when T_8 was deleted entirely, the k_{obs} was noticeably reduced (to 46; Figure 6C). However, the Y_m for all three mutants was unchanged. On the basis of the 3-fold difference between the k_{obs} of the abasic and C3 spacer mutants and that of the deletion mutant, we cautiously propose that the most optimal spatial arrangement at this location is a baseless sugar ring or simply a C3 spacer.

Structural Plasticity of 8−17 and 10−23. One particularly interesting discovery is that the substitution with an abasic nucleotide or a C3 spacer at *many* locations of both 10−23 and 8−17 does not eliminate the catalytic activity. This was observed for 4 out of the 9 tested locations in 8−17 and 11 out of the 15 tested positions in 10−23. This is a surprising finding given the sheer small size of the catalytic core (15 nucleotides each). This observation suggests that both DNAzymes have considerable structural plasticity to maintain their catalytic function.

Comparing the Catalytic Cores of 10−23 and 8−17: Is 10−23 a Special Variation of 8−17? It was previously proposed that the catalytic site of 10−23 is located at the two borders of the catalytic core sequence, specifically G_1, G_2, C_3, T_4, A_5, and G_6 at the 5′ border and C_{13} and G_{14} at the 3′ border (*45*). The results obtained in this study with the use of the abasic nucleotide and the C3 spacer, together with what we have already known about the 8−17 DNAzyme, raise the following question: Is 10−23 a special variant of 8−17?

Several lines of evidence support this speculation. First, as observed in this study and previous reports made by us and others (*15*, *18*, *19*, *21*, *37*, *44*−*46*), both DNAzymes share four identical, extremely conserved nucleotides arranged into two dinucleotide elements in AG followed by CG.

Second, the nucleotide that follows the conserved AG element in both DNAzymes is deoxycytidine (C_8 in 8−17 and C_7 in 10−23). More importantly, as we found in this study, the cytosine on this nucleotide appears to make important but not essential contribution to the activity of each DNAzyme: with removal of this base and retention of the pentose ring, both DNAzymes experience a significant drop in k_{obs} (from 100 to 5 in 8−17 and from 100 to 7 in 10−23; see Figures 4B and 6B).

Third, the two nucleotides flanking the conserved CG element are also identical in both DNAzymes, namely, A_{12} and A_{15} in both 8−17 and 10−23. The adenine base on each of these nucleotides, as inferred in the current study, appears to make an important (but nonessential) contribution to the DNAzyme function (as reflected by the observation that their removal caused a significant decrease in k_{obs}; see the data present in Figures 4C and 6B).

Finally, one of the key structural distinctions of the classic 8−17 is the existence of three-bp internal stem; however, two previous studies by our group (*15*, *37*) have found that even this structural element can tolerate substantial mutations because we have observed variations with one or two mismatch pairs or a bulged nucleotide. One particular construct, named 8−17NC, for example, contains only two A-T pairs flanked by a C residue on one side and A and G residues on the other (*37*). In light of these findings, we speculate that the nucleotides $G_1G_2C_3T_4$/$T_8A_9C_{10}A_{11}$ of 10−23 may create a tertiary structural element that closely resembles the three-bp internal helical element of 8−17 (Figure 7C). The formation of this structural element may also be the reason behind the observation of the slightly improved activity of the mutant construct of 10−23 in which the base of T_8 is removed, simply because this manipulation may create a better orientation of the conserved A_5G_6 in the folded active site. This may also explain why all other nucleobases (i.e., those on G_1, G_2, C_3, T_4, A_9, C_{10}, and A_{11}), and perhaps pentose rings on G_1, G_2, and C_3, make positive contributions to the optimal DNAzyme activity because each of these moieties may contribute specific molecular interactions (such as that are part of the foundation of the proposed helical-like element).

On the basis of the above analysis, we designed a chimeric DNAzyme construct, Chimera-1 (Figure 8A) where the sequence of the catalytic core of 10−23WT was altered in the following way: mutating $C_3 \rightarrow T$, $T_4 \rightarrow C$, $A_9 \rightarrow G$, $C_{10} \rightarrow A$, and inserting a C residue before A_{11}. Thus, Chimera-1 can be regarded as a chimeric DNAzyme that partially resembles both 8−17 (with its signature three-bp internal stem) and 10−23 (with the retention

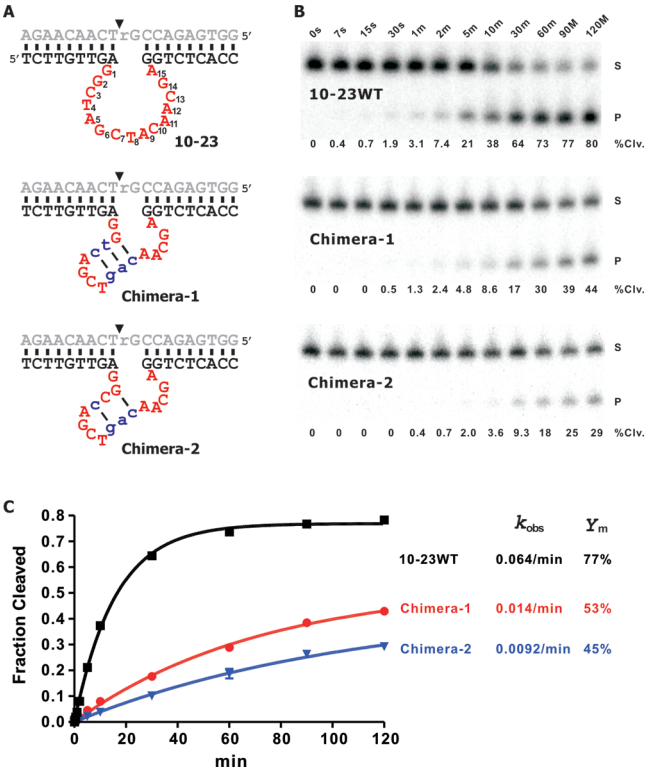

FIGURE 8: (A) The secondary structure, (B) PAGE-based assays, and (C) kinetic plots of 10−23 wild type and two chimeric DNAyzme constructs.

of 10 of the original 15 nucleotides in its catalytic core). As a control experiment, we also created a second construct, Chimera-2, in which one less mutation was made (i.e., without $C_3 \rightarrow T$ mutation) to weaken the internal stem. We expected that Chimera-1 would exhibit an activity similar to that of 10−23WT but considerably higher than that of Chimera-2. As shown in Figure 8B,C, Chimera-1 does display a fairly strong activity, with a k_{obs} of 0.014 min^{-1} and a Y_m of 53%. However, Chimera-1 is noticeably weaker than that of 10−23WT (k_{obs} = 0.063 min^{-1} and Y_m = 77%) and only slightly more active than Chimera-2 (k_{obs} = 0.0092 min^{-1} and Y_m = 45%). These results are not unambiguous enough to support the suggestion that 10−23 is a special variant of 8−17. However, we believe the results do not rule out such a possibility either because (1) the reduced activity of Chimera-1, compared to 10−23WT, may simply mean that the engineered stem in Chimera-1 does not provide a tertiary structural arrangement equivalent to that of 10−23WT, and (2) the similar catalytic activities of Chimera-1 and Chimera-2 may be explained by the existence of G_1 and T_8 in the sequence of 10−23WT but not 8−17WT, which may contribute to the formation of the tertiary structure of 10−23WT, thereby reducing the DNAzyme's dependence on the internal stem. Verifying or disapproving our proposal requires further investigations including the determination of high-resolution structures of 8−17 and 10−23, which is beyond the scope of the current study.

Significance of Abasic and C3 Spacer Based Mutation Approach. Mutational analysis through substitution of one natural nucleotide with another or deletion of the entire nucleotide is a well-adopted approach to scan the functional relevance of individual nucleotides in the sequence of a DNAzyme (or any other functional nucleic acid). Such an approach can reveal highly useful functional information such as nucleobase conservation, minimal sequence, and Watson−Crick base pairings that define the secondary structure of the DNAzyme. This simple approach, often combined with reselection experiments to isolate mutant DNAzymes with multiple nucleotide mutations, has facilitated the characterization of many DNAzymes, including 8−17 (*15*, *19*, *37*, *43*) and 10−23 (*45*, *46*). Another method is to substitute a nucleotide (whose importance has already been inferred by other methods) with unnatural nucleotide analogues to assess the importance of relevant functional groups on selected nucleotides to the function of a DNAzyme. Such an approach has been applied to study selected nucleotides in both 8−17 (*43*) and 10−23 (*45*). For example, by this method, N7 of adenine at A_6 and the 6-keto group of guanine at G_7 have been inferred as crucial functional groups in the catalytic activity of 8−17 (*43*). The lack of high-resolution structures for any known DNAzyme makes all mutagenesis approaches even more valuable as they can help to decipher the molecular mechanisms behind DNAzyme-mediated catalysis.

Since the nucleobase and the pentose ring are the two key components of a nucleotide in a DNAzyme, we believe the approach of removing the nucleobase (through the incorporation of abasic nucleotide) and then the ring structure (by inserting a C3 spacer) can also be a useful method to probe the relevance of these components from a given nucleotide to the catalytic activity of the DNAzyme. To our knowledge, such an approach has never been used to perform the functional analysis of any DNAzyme. The approach adopted in our study can offer insights in three aspects. First, it can define the functional contribution of the nucleobase and sugar ring of a nucleotide more clearly than other mutagenesis methods. This point is illustrated by the results observed with the abasic and C3 and deletion mutants at T_8 of 10−23 (Figure 5B). If only the deletion mutant were analyzed, the data would have been interpreted as that T_8 nucleotide makes a small but favorable contribution to the function of 10−23. However, when the abasic and deletion mutants were analyzed together, the interpretation can be expanded as that the nucleobase of T_8 does not make any contribution to the function of 10−23 but the sugar moiety contributes to the most optimal function of the DNAzyme. With the inclusion of the C3 spacer mutant, it becomes clearer that what is really needed at the location of T_8 is a small physical spacer to support the most optimal DNAzyme activity.

Second, the approach of component modification (first abasic substitution, then C3 spacer substitution, and finally deletion) makes it possible to resolve the functional contribution (or lack of it) of the nucleobase and the sugar moiety of a given nucleotide; this resolution would be more difficult using other types of mutational analysis. For example, four sugar rings were suggested in our study to make very small, if any, positive contributions to the function of 10−23 (the purple "s" letters in Figure 7B). Their inferences are made based on the small differential activity between a related set of the abasic and C3 spacer mutants.

Third, the adopted mutagenic analysis can generate a completely new set of results to support findings obtained with other mutagenesis methods. For example, the results obtained by our method for the 8−17 DNAzyme verified the previous findings that nucleobases on A_6, G_7, C_{13}, and G_{14} are extremely important for the function of the 8−17 DNAzyme and $T_{2.1}$ is highly important for the cleavage of rG-G junctions (*15*, *18*, *37*). Since functional analysis by any mutagenesis method (including the one featured in this study) is an indirect and "low-resolution"

method, a result by a particular approach could simply be an artifact but attributed to the function of the DNAzyme. The same result by different approaches makes the functional assignment of a selected nucleotide more reliable.

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SUPPORTING INFORMATION AVAILABLE

PAGE-based assays and kinetic plots of 8−17 mutants with abasic substitution at G_7, C_{13}, G_{14}, and $A_{15.0}$ (Figure S1); PAGE-based assays for 10−23WT and 10−23 mutants with abasic substitution at G_1, G_2, C_3, T_4, A_5, G_6, and C_7 (Figure S2), for 10−23 mutants with abasic substitution at T_8, A_9, C_{10}, A_{11}, A_{12}, C_{13}, G_{14}, and A_{15} (Figure S3), for 10−23 mutants with C3 spacer substitution at G_1, G_2, C_3, T_4, C_7, T_8, A_9, C_{10}, A_{11}, A_{12}, A_{15}, and with deleted T_8 (Figure S4), and for selected DNAzyme mutants in 24 h reaction time (Figure S5); k_{obs} and Y_m values for all of the 8−17 and 10−23 variants examined (Tables S1 and S3); relative k_{obs} and Y_m values for all of the 8−17 and 10−23 variants examined (Tables S2 and S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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