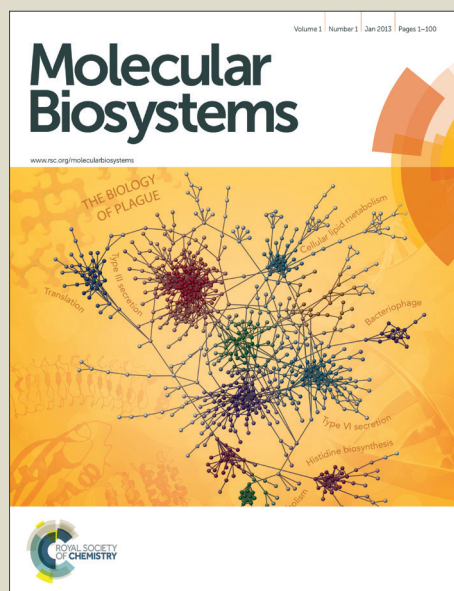


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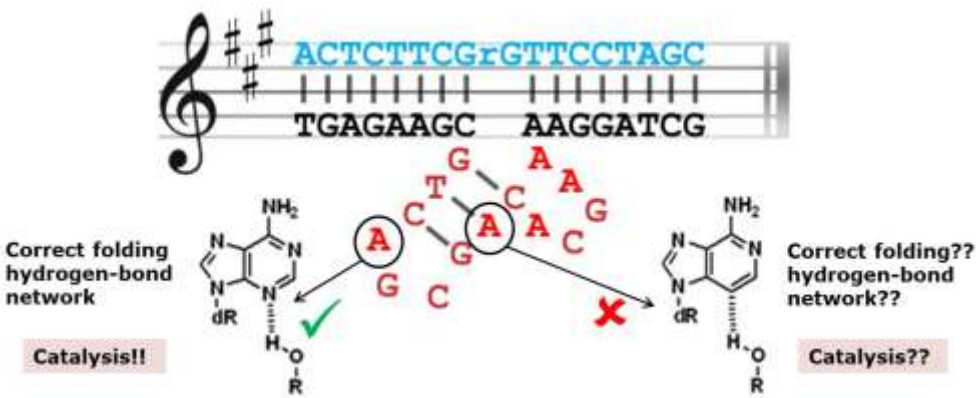
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ARTICLE

Probing the effect of minor groove interactions on the catalytic efficiency of DNazymes 8-17 and 10-23

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DNazymes (Dz) 8-17 and 10-23 are two widely studied and well characterized RNA-cleaving DNA catalysts. In an effort to further improve the understanding of the fragile interactions and dynamics of the enzymatic mechanism, this study examines the catalytic efficiency of minimally modified DNazymes. Five single mutants of Dz8-17 and Dz10-23 were prepared by replacing the adenine residues in the corresponding catalytic cores with 3-deazaadenine units. Kinetic assays were used to assess the effect on the catalytic activity and thereby identifying the importance of hydrogen bonding emanating from the *N*3 atoms. The results suggest that modifications at A₁₅ and A_{15,0} of Dz8-17 have a significant influence and show a reduction in catalytic activity. Modification at each location in Dz10-23 results in a decrease in the observed rate constants with A₁₂ appearing to be the most affected with a reduction of ~80% of *k*_{obs} and of ~25% of the maximal cleavage rate compared to the wild-type DNzyme. On the other hand, modification of A₁₂ in Dz8-17 showed a ~130% increase in *k*_{obs}, thus unraveling a new potential site for the introduction of chemical modifications. A pH-profile analysis showed that the chemical cleavage step is rate-determining, regardless of the presence and/or location of the mutation. These findings point towards an importance of the *N*3-nitrogens of certain adenine nucleotides located within the catalytic cores of the DNazymes for efficient catalytic activity and further suggest that they might directly partake in maintaining the proper tertiary structure. Therefore, it appears that minor groove interactions constitute an important feature of DNazymes as well as ribozymes.

Introduction

DNazymes (Dz or DNA enzymes) are single stranded DNA molecules, which are able to catalyze chemical reactions.¹⁻⁵ Since the first discovery of these catalytic DNA molecules by Gerald Joyce and Ronald Breaker in 1994 by application of *in vitro* selection techniques,⁶ a broad range of DNazymes was discovered, catalyzing a variety of reactions including RNA-^{7, 8} and DNA-cleavage,^{9, 10} DNA-mediated ester bond cleavage,¹¹ phosphomonoester hydrolysis of amino acid side chains,¹² C-C bond formation,¹³ generation of nucleopeptide linkages,¹⁴ formation of linear, branched or lariat RNA,^{15, 16} DNA phosphorylation¹⁷ and labelling,¹⁸ and thymine dimer photoreversion.¹⁹ Thus, DNA molecules can be coerced into catalyzing a broad array of reactions that involve not only oligonucleotide-based substrates where Watson-Crick base pairing dictates the interaction of the enzyme and the substrate, but also non nucleotidic substrates. Moreover, the inherent folding properties of DNA, its intrinsic capacity for replication, along with the cheap and efficient production of DNA oligonucleotides make DNazymes alluring targets for potential applications in numerous fields including chemical biology, biotechnology, and medicinal chemistry.^{20, 21} However, despite the advantages of generating DNazymes via *in vitro* experiments, there are also major drawbacks to these catalytic entities, especially when considering *in vivo* treatments and

medical applications. Indeed, like any other oligonucleotides, DNazymes are susceptible to degradation by nucleases. Moreover, efficient catalysis (*k*_{cat}/*K*_M values ≥ 10⁸ min⁻¹M⁻¹) usually relies on the presence of high concentrations of metal cofactors (M²⁺) exceeding the levels present in a cell.²²

In order to alleviate these drawbacks, chemical modifications can be incorporated into the scaffold of DNazymes. Efforts towards this goal usually proceed either by 1) including modified nucleoside triphosphates (dN*TPs) in combinatorial selection experiments²³⁻³² or 2) by incorporating nucleoside analogs into the catalytic core or binding arms of selected unmodified DNazymes via solid-phase oligonucleotide synthesis.³³⁻⁴¹ The advantage of solid-phase synthesis is that the chemical nature (and thus the purpose of the modification), the location, and the exact number of analogs can be easily modulated. However, since all combinatorial evolution experiments including SELEX follow the “you get what you select for” principle,⁴² DNazymes display finely tuned catalytic surfaces where even minute alterations can lead to a loss of activity.²⁷ Consequently, incorporation of modified nucleotides into the scaffold of DNazymes is a difficult undertaking since an investigation needs to be done to assess the effect that the chemical functionalities might have on the catalytic efficiency. Therefore, investigations on the mechanism and the dynamics of DNazymes and their catalytic core in particular, are of great interest.⁴³⁻⁴⁵

In this context, Dz8-17 and 10-23 are two well-known and widely used RNA-cleaving DNAzymes consisting each of a catalytic core with 15 nucleotides and two substrate binding arms (Fig. 1).⁷

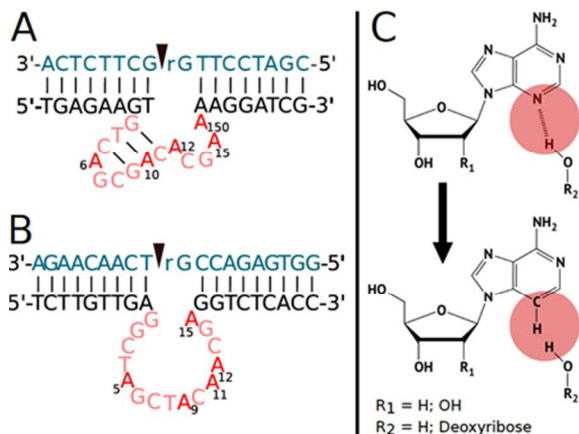


Fig 1 Hypothetical secondary structures of Dz8-17 (A) and Dz10-23 (B). Both have a 15nt catalytic core (shown in red) and two substrate binding arms (black). The substrate (blue) is bound via Watson-Crick base-pairing and cleavage occurs at the scissile riphodiester linkage (arrow). C) A carbon atom is incorporated at the 3-position of the purine ring, preventing the formation of hydrogen bonds.

Dz8-17 possesses a stem-loop motif in the catalytic core and mutagenesis experiments revealed that certain nucleotides such as A₆, G₇, and G₁₄ were strictly conserved and could not be altered or substituted, while other residues, such as A₁₂ and A₁₅ could be varied without causing a severe depletion in catalytic efficiency.^{8, 46, 47} Similarly, even though Dz10-23 seems to be devoid of structural motifs, various residues of the catalytic core have been shown to be highly conserved (such as G₁, A₅, and G₆), while other locations (T₈ and A₉ particularly) are thought to be tolerant to rather important modifications.^{38, 47-49} Recently, Li *et al.* have refined the understanding of the critical positions by incorporating an abasic site and a C3 spacer within the catalytic cores of both DNAzymes.⁴⁷

In the present study, the goal was to hone the understanding of the fragile interactions and dynamics of the enzymatic mechanism by focusing on other interactions involving nucleotides. Indeed, next to the well-known Watson-Crick and Hoogsteen base pairings, the N3-nitrogen in the purine ring of a guanine and even more importantly in adenine, is involved in the formation of the so-called A-minor interactions (adenine minor groove interactions). These interactions were first discovered in the secondary structures of rRNAs⁵⁰ and it could be shown that the A-minor motif in RNA is very common and mostly consists of an unpaired adenine inserted into the minor groove of RNA helices with a high C-G content.⁵¹⁻⁵³ A-minor motifs help to stabilize interactions between RNA duplexes, contacts of loops and helices or other conformations since the N3-nitrogen can act as a hydrogen bond acceptor that can facilitate interactions with both the 2'-OH groups and exocyclic amines of guanine or cytosine nucleotides.⁵² Studies carried out by McLaughlin *et al.* focusing on the N3-nitrogens of conserved adenine nucleotides in the hammerhead ribozyme, a naturally occurring ribozyme catalyzing the self-cleavage of RNA substrates, showed that A-minor interactions play an important role in ribozymes and are crucial for maintaining the enzymatic function.⁵⁴ Even though no A-minor motifs have

formally been identified in DNA so far, adenine N3-nitrogen atoms have been shown to play a crucial role in the hydration pattern of the minor groove of DNA (by hydrogen bonding to the primary water layer) and thus in the overall structure and stability of DNA duplexes.^{55, 56} Herein, we systematically replaced all the adenine units of the catalytic cores of the 8-17 and 10-23 DNAzymes with 2'-deoxy-3-deazaadenosine units, an analog that is devoid of a site that allows for hydrogen-bonding in the minor groove. Such a substitution caused a reduction of the observed rate constants when introduced at specific locations of both DNAzymes, suggesting that minor groove interactions play an active role in the mechanism of DNA-based catalysts.

Results and discussion

Nucleotide substitution in the catalytic core and thermal denaturation experiments

In order to investigate on the effect of minor groove interactions on the catalytic efficiency of DNAzymes, the phosphoramidite of 2'-deoxy-3-deazaadenosine was synthesized according to a known synthetic pathway.^{56, 57} This nucleoside analog was then incorporated into the catalytic core of the 8-17 and 10-23 DNAzymes via standard automated solid-phase oligonucleotide synthesis *in lieu* of each of the natural adenine residues (see Supporting Information). Since numerous variants of both the 8-17 and 10-23 DNAzymes exist, all obtained by selection under different conditions or by reselection from enriched libraries,^{1, 58, 59} we focused on the species used by Li *et al.* for the sake of comparison (Fig. 1).⁴⁷ Thus, the wild-type Dz8-17 used in this study consists of a 15 nucleotide catalytic core and two substrate binding arms. The catalytic domain has a stem formed by three Watson-Crick base-pairs, a loop containing an AGC triplet and 5 unpaired nucleotides to complete the catalytic core (Fig. 1A), for a total of 5 adenine nucleotides (i.e. A₆, A₁₀, A₁₂, A₁₅, and A_{15.0}). Similarly, the wild-type Dz10-23 has also a 15 nucleotide catalytic core but built up of only unpaired nucleotides and no base-pairing motifs for a total of 5 adenine units (i.e. A₅, A₉, A₁₁, A₁₂, and A₁₅; Fig. 1B). The resulting mutants were named 817_X and 1023_X, where X refers to the position of the substituent within the catalytic unit (Fig 1).

In order to assess the effect of the substitution on the overall stabilization of duplexes, thermal denaturation experiments of the natural and modified oligonucleotides were carried out (Tables 1 and 2 and Supporting Information). To better estimate the effect of the deletion of the N3 atom, the sequences of Dz8-17 and 10-23 were paired with the corresponding complementary sequence⁶⁰ and the obtained *T_m* values are summarized in Table 1 for Dz8-17 and in Table 2 for the Dz10-23. We decided to measure the melting temperatures of modified oligonucleotides corresponding to both DNAzymes paired with the complementary strand instead of a non-cleavable substrate since the modifications are all located within the catalytic core. The inclusion of the modification had only a marginally destabilizing effect on the duplex stability ($\Delta T_m \sim 1.0$ °C/modification) in the case of Dz8-17, which compares favorably to other reports where stronger destabilization of duplexes were observed.^{55, 56, 56, 61} Surprisingly, the melting curves of the single strands also showed cooperative and reversible melting events (Table 1 and Fig. S2, Supporting Information). Indeed, *T_m* values at around ~50°C are consistently observed, with mutant 817_15

displaying the highest T_m value (57.2°C) while the lowest T_m value was observed for 817_12 (T_m = 45.7°C). These rather high T_m values might be ascribed to the destacking of secondary structures, most likely the thermal denaturation of the putative 3bp stem of the central trilloop.⁸ Indeed, very short hairpin systems consisting of only two G-C base pairs and a shear G·A base pair have been shown to form extraordinarily stable systems (T_m >70°C),^{62, 63} and therefore, the formation of the GTCAGCGAC stem-loop could account for the observed melting event.

On the other hand, the variations in T_m values obtained in the case of Dz10-23 are more marked than in the case of Dz8-17. Indeed, the modified strands cause a destabilization of 2°C to 6°C as compared to the unmodified system (Table 2) and is in par with what has been observed in different sequence contexts.^{55, 56} Thus, the introduction of single modifications in the Dz10-23 construct leads to a more pronounced effect than similar alterations brought to the core of Dz8-17. Most melting curves for Dz10-23 clearly show a second transition at around 40°C, which might be due to a change of the tertiary structure of the duplex and/or the single strand. As observed in the case of Dz8-17, the melting curves of the single strands of Dz10-23 show cooperative and reversible melting events, albeit with T_m values ~10°C lower than those observed for the Dz8-17 single strands. 1023_5 shows the most stable secondary self-structure (T_m = 41.0°C) while 1023_11 displays the least stable self-structure (T_m = 32.0°C). The second transition in the melting curves might be ascribed to the melting of a duplex formed by two single strands maintained together by the 6nt long palindromic sequence located within the catalytic core rather than any putative secondary structure as in the case of Dz8-17.⁶⁴

Table 1 T_m data of the natural and modified duplex and single strands of Dz8-17

Dz8-17	Duplex ^a		Single strand	
	Avg. T_m	ΔT_m	Avg. T_m	ΔT_m
817_WT	64.3°C	0.0°C	48.7°C	0.0°C
817_6	63.3°C	-1.0°C	52.2°C	3.5°C
817_10	63.3°C	-1.0°C	51.7°C	3.0°C
817_12	63.3°C	-1.0°C	45.7°C	-3.0°C
817_15	63.2°C	-1.0°C	57.2°C	8.5°C
817_150	64.0°C	-0.4°C	54.5°C	6.8°C

^acomplement: 5'-CGATCCTTTTCGTGTCGCTGACACTTCTCA-3'

Table 2 T_m data of the natural and modified duplex and single strands of Dz10-23

Dz10-23	Duplex ^a		Single strand	
	Avg. T_m	ΔT_m	Avg. T_m	ΔT_m
1023_WT	65.0°C	0.0°C	37.0°C	0.0°C
1023_5	63.0°C	-2.0°C	41.2°C	4.2°C
1023_9	62.0°C	-3.0°C	39.7°C	2.7°C
1023_11	62.3°C	-2.8°C	32.0°C	-5.0°C
1023_12	58.8°C	-6.2°C	35.7°C	-1.2°C
1023_15	60.7°C	-4.4°C	35.3°C	-1.7°C

^acomplement: 5'-CGATCCTTTTCGTGTCGCTGACACTTCTCA-3'

Effects of substitutions on the catalytic activity

In order to assess the effect of potential minor groove interactions on the catalytic activity, cleavage reactions were performed under single turnover conditions with a chimeric DNA/RNA substrate ³²P-radiolabeled with both the wild-type and modified constructs. The results of the kinetic assays are shown in Figure 2A for Dz8-17 and 2B for Dz10-23. In addition, the values for the observed rate constants (k_{obs}) and maximal cleavage rates (Y_m) were derived from curve-fitting of the data and the results are summarized in Table 3. The k_{obs} and Y_m values of all the modified constructs are normalized against those of the native DNazymes.

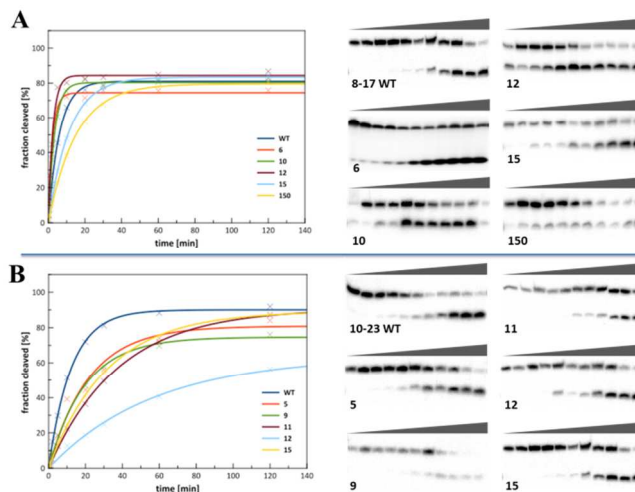


Fig 2 Kinetic plots and PAGE gels of the kinetic assays of the wild type and modified constructs. A) Dz 8-17. B) Dz 10-23.

For Dz8-17, it can be noted that the maximal cleavage yield for all constructs remains unchanged (Y_m = ~80%). The values of k_{obs} on the other hand vary substantially and depletions as well as improvements in the catalytic activity can be observed. Indeed, the catalytic efficiencies of three of the mutant DNazymes (817_6, 817_10, and 817_15) seem to be rather unaffected by the introduction of the modification into the catalytic core: mutants 817_6 and 817_10 display a slight rate improvement of ~40% as compared to wild-type DNzyme 8-17, while in the case of 817_12 a 2-fold improvement can be observed which compares favorably to other modified constructs. On the other hand, 817_15 and 817_150 show an important loss of catalytic activity since the k_{obs} values dropped by ~40-70%. Interestingly, while the introduction of a 3-deazaadenine residue at A_{15,0} caused the largest drop in rate constant, this location had been shown to be rather tolerant for other substitutions such as an abasic site or a C3-spacer.⁴⁷ However, the nonessential A₁₅ and A_{15,0} were shown to be responsible for the modulation of the cleavage site selectivity⁶⁵ and to be in close contact with the cleavage site nucleotides (i.e. rG-T, Fig 1A).^{1, 66} Thus, it seems plausible that the deletion of the N3-nitrogens has an impact on the contacts of the catalytic core with the substrate which may account for the drop in activity. In addition, A₆ located in the AGC loop has been shown to be a critically conserved residue involved in functionally important hydrogen bonds.^{46, 65, 67} However, A₆ appears not to be involved in important interactions via the N3

atom since the modification only causes a slight variation in k_{obs} .

The inclusion of 3-deazaadenine residues into specific locations of the catalytic core of Dz8-17 has an effect on the catalytic efficiency, which hints at a possible participation of the adenosine *N3*-nitrogens in the mode of action of Dz8-17 albeit not necessarily in the chemical cleavage step since all Y_m values are unchanged (*vide supra*). Taken together, substitution at positions A_6 , A_{10} and A_{12} of Dz8-17 has only a little impact on the catalytic activity while the *N3*-nitrogens at A_{15} and $A_{15,0}$ seem to be of importance for catalysis. Taking into account that A_6 , A_{10} and A_{12} are positioned within the stem-loop motif it seems reasonable that base-pairing is more important and outbalance the loss of the *N3*. On the other hand, A_{15} and $A_{15,0}$ are positioned in close proximity to the scissile guanosine (rG) and thus interactions with the 2'-OH of the substrate might be possible.

Table 3 k_{obs} and Y_m values for the wild type and modified constructs of Dz8-17 and Dz10-23

Dz8-17	k_{obs} (min ⁻¹)	Y_m	rel. k_{obs}	rel. Y_m
817_WT	0.220 ± 0.015	80.3 ± 1.2	1.00	1.00
817_6	0.303 ± 0.017	79.5 ± 1.0	1.38	0.99
817_10	0.313 ± 0.023	80.0 ± 1.1	1.42	1.00
817_12	0.520 ± 0.036	79.2 ± 1.1	2.36	0.99
817_15	0.142 ± 0.010	81.9 ± 1.0	0.65	1.02
817_150	0.070 ± 0.007	79.3 ± 2.5	0.32	0.99
Dz10-23	k_{obs} (min ⁻¹)	Y_m	rel. k_{obs}	rel. Y_m
1023_WT	0.103 ± 0.009	86.1 ± 2.1	1.00	1.00
1023_5	0.051 ± 0.008	86.2 ± 3.8	0.50	1.00
1023_9	0.050 ± 0.005	84.6 ± 2.6	0.49	0.98
1023_11	0.032 ± 0.005	87.0 ± 4.8	0.31	1.01
1023_12	0.017 ± 0.004	63.8 ± 6.8	0.17	0.74
1023_15	0.033 ± 0.003	88.3 ± 3.1	0.32	1.03

For Dz10-23 the maximal cleavage yields of all the modified constructs, except for the mutant 1023_12, reach the same percentage of cleavage as the unmodified DNAzyme ($Y_m \sim 85\%$). The maximal cleavage rate attained only 64% in the case of mutant 1023_12. Unlike what has been observed for Dz8-17, the rate constants of all the modified constructs are lower than that of wild-type Dz10-23. For 1023_5 and 1023_9 a rate depletion of around 50% is observable (Table 3). In the case of 1023_11 and 1023_15 a more marked decrease in rate constant ($\sim 70\%$) is observed, while the catalytic activity is substantially hindered in the case of 1023_12 since a drop of around 80% is observed.

Previous studies have shown that A_5 was a critical residue for maintaining the catalytic activity even though an $A_5 \rightarrow C_5$ mutation was tolerated and it was suggested that one of the nitrogen atoms of the nucleobase was involved in an important hydrogen bond interaction.⁴⁷⁻⁴⁹ However, our results ($k_{\text{obs}}(\text{rel}) = 0.5$ for A_5) and a previous report⁴⁸ indicate that none of the amines located at positions 3 and 6, respectively, play a critical functional role. A_9 has been found to be rather tolerant to substitution and modification,^{39, 47, 68} which is reflected by the small, ~ 2 -fold decrease in rate constant upon substitution with 3-deazaadenine. Moreover, A_{11} was deemed to be merely

serving as a physical spacer,⁴⁷ but the rather important reduction in k_{obs} ($k_{\text{obs}}(\text{rel}) = 0.31$) suggests that A_{11} might be playing a role in the folding of the DNAzyme into its catalytically active conformation. Deletion of the *N3* atom at A_{15} , located at the 3'-end of the catalytic core in proximity to the scissile bond, causes the catalytic activity to drop to a similar extent as for A_{11} ($k_{\text{obs}}(\text{rel}) = 0.32$) even though this base is not thought to be quintessential for catalysis.⁴⁷ Indeed, substitution of A_{15} with G or T⁴⁸ or with a 7-deazaadenine analog equipped with an amino acid side-chain⁶⁹ led to fully active constructs, while the ablation of this nucleobase (abasic site) generated a construct with a very low k_{obs} but that maintained its catalytic activity (i.e. a high Y_m value).⁴⁷ Finally, substitution at A_{12} causes a significant diminution of the rate constant ($k_{\text{obs}}(\text{rel}) = 0.17$) and 1023_12 is the sole mutant that also presented a diminution of the maximum cleavage yield ($Y_m(\text{rel}) = 0.74$), hinting at an implication of the *N3* atom of this nucleobase in the catalytic mechanism. This is surprising since reports where A_{12} was substituted with any of the other natural nucleobases⁴⁸ or with 8-aza-7-deazaadenine analogs⁶⁸ clearly showed that A_{12} was probably not critical for catalytic activity. However, we can suggest that the reduction of the observed rate constant might be consistent with the disruption of one stabilizing hydrogen bond of the transition state since such an event has been estimated to lower the stability by 0.5-2 kcal/mol which in turn causes a three- to 25-fold reduction of the reaction rate.^{46, 70} Thus, the *N3* atom at A_{12} of the catalytic core might be involved in critical interactions and might be quite important for catalysis which is reflected by a strong decrease in both k_{obs} and Y_m . Taken together, it appears that the *N3* atoms of A_{11} , A_{12} , and A_{15} all have an effect on the observed rate constants and thus might be involved in the maintenance of the adequate tertiary structure rather than influencing or participating in the chemical cleavage step.

pH-rate profiles

In order to further investigate the influence of the deletion of the *N3* atom on the catalytic efficiency, two modified DNAzymes, namely 817_12, 817_150, 1023_5 and 1023_12 along with the parent DNAzymes were used for a pH-rate profile analysis. The same kinetic assays as described in the preceding section were carried out at different pHs (namely 6, 6.5, 7, 7.5, 8 and 8.5). The cleavage efficiency was measured as described above and the values for k_{obs} and Y_m were obtained by curve-fitting. The pH rate profiles are shown in Figures 3, S5 and S6, while the measured k_{obs} and Y_m values are summarized in Table S9 (Supporting Information). The wild type constructs as well as the modified DNAzymes follow a similar trend since an increase in k_{obs} with increasing pH is observed for Dz8-17 as well as for Dz10-23. The plotted values result in the typical linear log pH-rate profiles with a slope of ~ 1 for all the investigated Dz8-17 constructs (Fig. 3A and Table S10), and a slight plateau is observed at $\text{pH} \geq 8$ which might arise from a weaker binding of the substrate at higher pH values as observed for different 8-17 variants^{61, 71, 72} and the hammerhead ribozyme.⁷³ The preservation of the linear relationship in the pH-rate profiles of all the Dz8-17 constructs suggests that the chemical cleavage step (i.e. transesterification of the embedded labile riboguanosine linkage) rather than a putative conformational change is the rate-determining step as in the case of the parent Dz8-17.^{54, 72-74} All the 8-17 DNAzymes that were investigated displayed k_{obs} values (2.5-5.0 min⁻¹) at pH 8.5 which are considerably higher than the values at pH 7.0.

Moreover, the Y_m values for wild-type Dz8-17 and 817_12 remained unchanged throughout the entire pH range, while mutant 817_150 showed a marked increase in Y_m value with increasing pH (from ~50 to ~80%). This observation further confirms that the substitution of the $N3$ -atom by a carbon atom on adenine at position A_{12} seems to be uncritical and that the $N3$ has no direct effect or involvement in the cleavage process.

Furthermore, this site might be suitable for the inclusion of additional functional groups in order to improve the catalytic efficiency, since higher k_{obs} values are invariably observed. In contrast, the $N3$ at position $A_{15,0}$ seems to be of importance for the proper catalytic process since there is a clear attenuation of the cleavage efficiency.

A similar trend was observed for Dz10-23 except for 1023_12 where a break in linearity was detected (Fig. 3B). Indeed, k_{obs} increases steadily with pH for the wild type as well as for constructs 1023_5 and 1023_12 and the values of the observed rate constants are higher at pH 8.5 than at 7.0, albeit the change is more marked (~15-fold increase) for wild-type than for the modified constructs (2-7 fold increase). This is consistent with the pH-rate profiles reported for wild-type Dz 10-23^{74, 75} and modified variants.⁶⁸ Unlike all other modifications, the curve for construct 1023_12 did not show any linearity but still a clear pH dependency (the pH-rate profiles for Dz1023_12 are the result of five independent experiments). This very distinct curve, compared to the other constructs, might be explained by the protonation state of the nucleobase.

We hypothesize that the protonation state of the nucleobase at position A_{12} is of importance and plays a crucial role in the enzymatic activity. Indeed, by exchanging the $N3$ -nitrogen with a carbon atom the pK_a is shifted from ~4.0 to ~7.0^{76, 77} which could explain the very low k_{obs} values observed at pH 6 and pH 6.5 and the drastic increase at pH 7 (~20 fold). In addition, mutant 1023_12 displays a combination of reduced maximal cleavage yield and reduced k_{obs} values which might point towards a structural change within the DNAzyme scaffold and therefore might be a hint for minor groove interactions emanating from the $N3$ at this position. Taken together, it seems that the two investigated constructs of Dz10-23 have a need for the $N3$ atoms for their catalytic activity and might be involved in transition state stabilization and/or hydrogen bonding with the coordinated inner sphere water molecules of the Mg^{2+} cation cofactor.^{54, 78, 79}

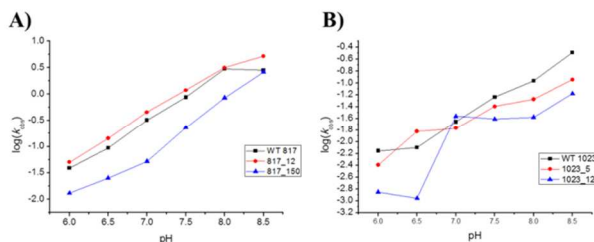


Fig 3 pH dependence of the observed rate constant for wild-type and mutant DNAzymes. A) Dz 8-17; B) Dz 10-23

Conclusions

The presence of the $N3$ -nitrogen atoms of specific residues in the catalytic cores of Dz8-17 and Dz10-23 seems to be of importance for the catalytic activity. The removal of the $N3$ -atoms in some of the conserved adenine nucleobases alters the observed rate constants of both DNAzymes that were

investigated. Thus, minor groove interactions seem to play a role in DNAzymes, albeit to a lesser extent than to what has been observed in their RNA counterparts.⁵⁴ Indeed, when the hammerhead ribozyme was subjected to a similar analysis, the deletion of the $N3$ atom of $A_{15,0}$ led to a drastic (55-fold) drop of the catalytic activity,⁵⁴ highlighting the importance of hydrogen bonds, hydration and metal bonding to the $N3$ atoms, which are present in many RNA folds.⁵²

When A_{15} and $A_{15,0}$ were modified in the catalytic core of Dz8-17, a significant loss in catalytic activity was observed. On the other hand, in Dz10-23 all modifications showed a reduction in the observed rate constant but A_{12} was found to be the most affected with an ~80% reduction of activity. Surprisingly, also rate enhancement could be observed for some modifications such as A_{12} in Dz8-17, thus unraveling a new site for the appendage of functionalities.

The structural features responsible for metal-specificity and binding in DNAzymes have still not fully been identified.⁸⁰ On the other hand, $N7$ and $N9$ sites have been shown to be important metal binding sites in various nucleic acids, especially in ribozymes.^{81, 82} In addition, adenine analogs have been used to highlight the coordination potential of more unusual binding sites such as the $N3$ nitrogens of adenine.^{83, 84} Whilst our data does not suggest a direct binding of the Mg^{2+} cations to $N3$ -nitrogens of adenine, these atoms could be involved in coordination to the 2'-OH units of the scissile residues of the substrates (especially $A_{15,0}$ in Dz 8-17), to water molecules coordinated in the innersphere of the Mg^{2+} cofactor, or of water molecules that are part of the ribbon of hydration.⁸⁵ The identification of critical positions – together with previously identified important nucleotides within the catalytic cores of Dz8-17 and Dz10-23 – reinforces the assumption that the network of hydrogen bonding and other interactions are a fragile structure, influenced by many parameters and that play an important role in the catalytic mechanism of nucleic acid DNAzyme enzymes. The generality of these observations still needs to be extended to other catalytic DNAs (e.g. the bipartite⁸⁶ or 614⁸⁷ DNAzymes) and work towards this aim as well as more detailed kinetic investigations (e.g. to assess the effect of metal cofactors, the role of the $O2$ functionalities of dT residues⁸⁸) are currently underway. Nonetheless, this study reveals that these 'A-minor like' interactions might be regarded as another protagonist within the catalytic mechanism of DNAzymes.

Experimental

General procedures

All chemicals and solvents used were purchased from Sigma, AK Scientific Inc. or Alfa Aesar, unless stated otherwise. All reactions were performed under Ar in flame-dried glassware. Anhydrous solvents for reactions were obtained by filtration through activated aluminum oxide, or by storage over 4Å activated molecular sieves. Flash chromatography was performed using SiliaFlash®P60 (230–400 mesh) from Silicycle. Thin layer chromatography was carried out on precoated glass-backed plates of silica gel (0.25mm, UV₂₅₄) from Macherey-Nagel. NMR spectra were recorded at RT on a Bruker DRX-400 or a Bruker AC-300 spectrometer (400 or 300 MHz for 1H , 101 or 75.5 MHz for ^{13}C , 121.4 MHz for ^{31}P , and 376.5 MHz for ^{19}F) and all spectra were referenced to the signals of the corresponding solvent. Chemical shifts are given in ppm (δ scale) and coupling constants (J) in Hz. Multiplicities

are abbreviated as follows: s= singlet, d= doublet, t= triplet, q= quadruplet, m= multiplet, br= broad. High resolution electrospray ionization (ESI) mass spectra (MS, m/z) were recorded on a Thermo Scientific LTQ Orbitrap XL instrument. MALDI-TOF spectra were recorded on an Applied Biosystems Sciex QSTAR Pulsar instrument. Natural nucleoside phosphoramidites were purchased from Tides Service Technology and the solid supports were from Glen Research.

For the determination of the concentration of the oligonucleotides a Perkin Elmer Lambda 16 UV/VIS spectrometer was used. The concentrations were obtained by using the absorbance at 260nm (A_{260}). Thermal denaturation experiments were carried out on a Varian Cary 3E UV/VIS spectrometer. Absorbances were monitored at 260nm. The heating/cooling rate was set to 0.5°C per minute and all samples were covered with mineral oil to prevent evaporation of the solution during the heating process. The program was set to a heating-cooling-cycle starting at 25°C and heating to 90°C. The first derivatives of the melting curves were calculated by the Variant software. The phosphorimager plates were analyzed on a Storm 820 Phosphorimager from Amersham Molecular Devices with the ImageQuant software (both from GE Healthcare). Unmodified oligonucleotides were purchased from Microsynth and gel purified (PAGE 20%). Acrylamide/bisacrylamide (19:1, 40%) was obtained from Serva. T4 Polynucleotide kinase was purchased from Promega and γ - 32 P-ATP from Hartmann Analytics.

Synthesis of the phosphosphoramidite

The 3-deazaadenine nucleobase was synthesized following a literature procedure.⁵⁷ The preparation of the 3-deazaadenine phosphoramidite was carried out by a variation of a literature procedure.⁵⁶ Briefly, to a flask containing the DMTr-protected precursor (100 mg, 0.15 mmol, 1 eq.) dissolved in ACN (3 mL), *N*-ethyl-diisopropylamine (0.075 mL, 0.55 mmol, 3 eq.) was slowly added, followed by a dropwise addition of 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (0.048 mL, 0.28 mmol, 1.5 eq.). The reaction was stirred at RT until TLC showed complete conversion of the precursor. The reaction was then extracted with NaHCO_3 , filtered, and dried over MgSO_4 . Volatiles were evaporated *in vacuo*. The white solid was purified by flash column chromatography eluting with EtOAc:Hexanes 6:4 + 1%TEA to afford the 3-deazaadenine phosphoramidite in 50% yield (65 mg). Data for the 3-deazaadenine phosphoramidite:

$^1\text{H-NMR}$ (300 MHz, CDCl_3) δ 8.14 (dd, $J = 5.6, 4.2$ Hz, 1H), 8.10 (s, 1H), 7.63 (d, $J = 5.6$ Hz, 1H), 7.40 (d, $J = 7.5$ Hz, 2H), 7.27 (ddd, $J = 12.2, 5.7, 2.6$ Hz, 9H), 6.83 – 6.77 (m, 4H), 6.31 – 6.23 (m, 1H), 4.80 – 4.72 (m, 1H), 4.32 – 4.24 (m, 1H), 3.77 (d, $J = 1.3$ Hz, 7H), 3.72 – 3.53 (m, 4H), 3.45 – 3.36 (m, 2H), 2.67 – 2.59 (m, 3H), 2.46 (t, $J = 6.3$ Hz, 1H), 1.37 (s, 12H), 1.21 – 1.16 (m, 10H), 1.11 (d, $J = 6.8$ Hz, 3H). $^{31}\text{P-NMR}$ (122 MHz, CDCl_3) $\delta = 149.29, 149.27$ ppm. HR-MS (ESI⁺) m/z : calc. for $\text{C}_{49}\text{H}_{59}\text{N}_6\text{O}_8\text{P}$ ($\text{M} + \text{H}$)⁺: 891.4205; found: 891.4195.

Oligonucleotide Synthesis

All oligonucleotides were synthesized by standard solid-phase phosphoramidite chemistry on a 1.3 μmol scale using a Pharmacia LKB Gene Assembler Special DNA Synthesizer with 5-(ethylthio)-1*H*-tetrazole (0.25 M in ACN) as the activator and coupling times of 90 s for the natural phosphoramidites and 12 min for the 3-deazaadenine

phosphoramidite. The oligomers were deprotected using a 33% NH_3 solution for 0.5 h at room temperature followed by 16 h at 55°C in a screw cap 1.5 mL tube. The oligonucleotides were filtered through 13 mm Yeti HPLC filters (PTFE membrane, pore size 0.45 μm). Subsequently, the samples were precipitated with ethanol and purified by 15% polyacrylamide gel electrophoresis. Desalting was achieved using Sephadex G-10 spin columns.

Kinetic assays

The cleavage reactions were performed under single turnover conditions. The reactions were carried out in 10 μL HEPES buffer 10x (0.5 M HEPES, 2 M NaCl, 100 mM MgCl_2 , pH 7.2 for Dz8.17 and 0.5 M HEPES, 2 M NaCl, 500 mM MgCl_2 , pH 8 for Dz10-23) at room temperature. Rather high $[\text{Mg}^{2+}]$ were chosen for Dz10-23, so that the rate constants are $>10^{-2} \text{ min}^{-1}$.⁴⁷ Indeed, when Dz10-23 is assayed at low $[\text{Mg}^{2+}]$ and under single-turnover conditions with DNA-RNA chimeric substrates, k_{obs} are usually comprised in the 10^{-2} - 10^{-3} min^{-1} range.^{38, 39} A mixture of 60 pmol of DNAzyme, 10 μL buffer and H_2O was prepared in an Eppendorf tube and pre-heated at 37°C. 2 μL of ^{32}P -radiolabeled substrate was mixed with 0.5 pmol of the unlabeled substrate and also incubated at 37°C prior to the reaction. 2.5 μL of the substrate mixture were added to the reaction mixture to initiate the reactions in a total reaction volume of 100 μL . At each time point, 5 μL of the reaction mixtures were either quenched in an equal volume of stop solution (70% formamide, 30% EDTA 0.5 M, pH 8.0, 0.1% bromophenol, 0.1 % xylene cyanol) for Dz8-17 or in 5 μL EDTA 0.5M pH 8, followed by the addition of NaOAc (300 mM final concentration) and 500 μL EtOH and precipitation by centrifugation for Dz10-23. For Dz 8-17, Dz 10-23 and all modified versions time-points were taken after 7, 15, 30 s, 1, 2, 5, 10, 20, 30, 60, and 120 min. At least three individual experiments were conducted for each construct and the averaged results are given. The reactions were analyzed by 20% polyacrylamide gel electrophoresis. Visualization was carried out by means of a phosphorimager and polygons were drawn around the bands corresponding to the cleaved and uncleaved species using the ImageQuant Software. The obtained data was then fitted to first-order reactions with the OriginPro (version 8.0) Software using the equation $Y = Y_m(1 - e^{-kt})$, where k (k_{obs}) is the observed rate constant (in min^{-1}), Y the cleavage yield (in %) at any given time-point t , and Y_m the maximum cleavage yield. All errors are given as the averaged standard deviation from the exponential fit of each time-course.

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Notes and references

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