Sequence Requirements in the Catalytic Core of the “10-23” DNA Enzyme*

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A systematic mutagenesis study of the “10-23” DNA enzyme was performed to analyze the sequence requirements of its catalytic domain. Therefore, each of the 15 core nucleotides was substituted separately by the remaining three naturally occurring nucleotides. Changes at the borders of the catalytic domain led to a dramatic loss of enzymatic activity, whereas several nucleotides in between could be exchanged without severe effects. Thymidine at position 8 had the lowest degree of conservation and its substitution by any of the other three nucleotides caused only a minor loss of activity. In addition to the standard nucleotides (adenosine, guanosine, thymidine, or cytidine) modified nucleotides were used to gain further information about the role of individual functional groups. Again, thymidine at position 8 as well as some other nucleotides could be substituted by inosine without severe effects on the catalytic activity. For two positions, additional experiments with 2-aminopurine and deoxypurine, respectively, were performed to obtain information about the specific role of functional groups. In addition to sequence-function relationships of the DNA enzyme, this study provides information about suitable sites to introduce modified nucleotides for further functional studies or for internal stabilization of the DNA enzyme against endonucleolytic attack.

Ribozymes are catalytically active nucleic acids that can be designed to cleave target RNA molecules in a sequence specific manner. This opportunity has resulted in a large number of reports using ribozymes for target validation studies and potentially therapeutic applications (1, 2).

One of the most intensively studied catalytic RNA molecules is the hammerhead ribozyme. It was originally obtained from the RNA of plant pathogens but has subsequently been discovered in a variety of other genomes. The hammerhead was the first ribozyme to be divided into enzyme and substrate strands, making multiple turnover possible (3, 4). These findings led to design of numerous ribozymes that selectively bind to a target RNA by Watson-Crick base pairing and cleave phosphodiester bonds.

Major challenges for successful in vitro approaches, however, are efficient cellular uptake of the ribozyme and stability against nucleolytic degradation combined with high catalytic activity. These problems can be overcome by introducing modified nucleotides. This requires the knowledge of essential nucleotides in the catalytic core. Sequence requirements in the catalytic core of the hammerhead ribozyme were systematically studied and revealed that only one nucleotide of the catalytic core can be exchanged without a dramatic loss of activity (5). Furthermore, the importance of individual functional groups has intensively been studied by introducing modified nucleotides (6–9).

These studies finally led to the design of highly modified ribozymes with enhanced stability and a reasonable level of catalytic activity (10, 11). Five purine nucleotides within the catalytic core, however, must remain as unmodified ribonucleotides to maintain its catalytic activity. These positions are still susceptible to endonucleolytic attack.

A new generation of catalytic nucleic acids composed entirely of DNA has been obtained by in vitro selection using a combinatorial DNA library (12). Compared with synthetic RNA enzymes, DNA enzymes are easier to prepare and less sensitive to chemical and enzymatic degradation. The most prominent deoxyribozyme named “10-23 DNA enzyme” consists of a catalytic core of 15 nucleotides and two flanking arms of 6–12 nucleotides on either side. It is highly sequence-specific and generalizable to cleave almost any target RNA. The DNAzyme has been applied to suppress the expression of a variety of target genes (1, 2) and could successfully be used in animal models (13–15).

Despite numerous applications, however, only very little is known about mechanistic details of the 10-23 DNA enzyme. It was found to be almost intolerant toward variation, with the only exceptions known being a T → A or T → C exchange at position 8, a G → A exchange at position 14, and an A → G exchange at position 15 (16). Kinetics of cleavage were analyzed under conditions of varying pH, choice of divalent metal cofactor, and its concentration (17). A strong preference of the cleavage reaction was observed over ligation of the two cleavage products. A first crystallographic structure could be obtained, but it was unlikely to represent the catalytically active conformation (18). Thus, a systematic study about the function of individual residues is still lacking.

In the present study we investigated sequence requirements in the catalytic domain of DNA enzymes. We substituted each of the core nucleotides by each of the other naturally occurring nucleotides and analyzed the activity of mutant deoxyribozymes. Furthermore, modified nucleotides were introduced to study the importance of exocyclic functional groups.

**EXPERIMENTAL PROCEDURES**

Oligonucleotides—Unmodified oligodeoxynucleotides were obtained from MWG Biotech, Ebersberg, Germany. DNA oligonucleotides containing modified nucleotides (inosine, 2-aminopurine, and deoxypurine) were purchased from IBA GmbH, Göttingen, Germany.

The sequence of the DNA enzyme used was as follows: DNA enzyme V29 (898): TCT TGT TGA GCC TAG CTA CAA CGA GGT CTC ACC.

The PAGE-purified target RNA was also obtained from IBA GmbH, Göttingen, Germany (target RNA V29: GGU GAG ACC GUC AAC AAG A).

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For a control experiment a second DNAzyme and its target RNA with the following sequences were used: DNA enzyme V15 (9/9), ATG TCA TGA GGC TAG CTA CAA CGA GGT TAG GGC; target RNA V15, CCC CUA ACC GUC AUG ACA U.

Radioactive Labeling of Short Target RNA—For cleavage experiments with short target RNA 1 pmol of the oligonucleotide was labeled with 10 μCi of (γ-32P)ATP using T4 polynucleotide kinase (Promega, Madison, WI) for 90 min at 37 °C. The radioactive probe was purified by 12% denaturing polyacrylamide gel electrophoresis and was eluted from the gel with 0.3 M sodium acetate, pH 5.5, for 45 min at 80 °C. The RNA was then precipitated from solution overnight at −20 °C by the addition of 2.5 volumes of 100% ethanol.

In Vitro Transcription of Long Target RNA—The cDNA of the vanilloid receptor (VR1L1) was cloned as described previously (19). Prior to transcription the plasmid was linearized with KpnI. In vitro transcription was performed with the RibomAX Large Scale Production System from Promega (Madison, WI) according to the manufacturer’s instructions.

DNA Enzyme Cleavage Reaction—To analyze the catalytic activity of various DNA enzymes 1 pmol of unlabeled and 20,000 cpm of labeled short target RNA were incubated in the presence of a 10-fold excess of the DNA enzyme in 10 μl of ribonuclease buffer (10 mM MgCl2, 50 mM Tris-HCl, pH 7.5) at 37 °C for 20 min. Prior to the reaction, DNA enzymes and target RNA were denatured for 2 min at 85 °C and cooled down to 37 °C. Reactions were started by addition of EDTA to a final concentration of 50 mM and by cooling on ice. Samples were denatured for 5 min at 65 °C, and substrate RNA and cleavage products were separated on a 20% polyacrylamide gel. For gel evaluation a Molecular Dynamics Storm 840 Phosphoimager was used. Averages of two independent experiments are given, and the range of values obtained in each experiment is indicated.

DNA Enzyme Kinetics under Single Turnover Conditions—Single turnover experiments with a 100-fold excess of enzyme were performed for long target RNAs as described previously (19). Experiments were performed in 50 mM Tris-HCl and 10 mM MgCl2. DNA enzymes were denatured at 65 °C for 2 min prior to the reaction and cooled down to 37 °C. Reactions were started by adding the DNA enzyme to the substrate with a final concentration 100 nM. Aliquots were taken at different time points, and the reactions were stopped by the addition of 83 mM EDTA and cooling on ice. The cleavage reactions were analyzed by agarose gel electrophoresis. Band intensities were determined with the program Quantity One (Bio-Rad, Munich, Germany), and data were further analyzed by fitting with a single exponential decay function using Microcal Origin (Microcal, Northhampton, MA). All values given are the average of two independent experiments which differed by less than 10%.

RESULTS

In a previous study we could generate DNA enzymes cleaving the full-length VR1 mRNA with high efficiency (19). The DNAzyme against target site 29 and its complementary RNA are shown in Fig. 1A. The catalytic core is numbered in 5′ → 3′ direction. Fig. 2 gives an overview over the bases used in the present study.

Systematic Substitution of Core Nucleotides by Naturally Occurring Deoxynucleotides—Our first intention was to identify nucleotides in the catalytic core that can be substituted by any of the other naturally occurring deoxynucleotides without complete loss of catalytic function. To analyze a large number of modified DNA enzymes, we preferred single point measurements over a complete kinetic characterization. DNA enzymes containing a single mutated position in the catalytic domain were incubated with labeled target RNA (10-fold excess of the respective DNAzyme) for 20 min at 37 °C. Subsequently, the percentage of target RNA cleavage was determined and normalized to the activity of the unmodified 10-23 DNA enzyme. As can be seen in Fig. 3, positions at the borders of the catalytic domain have a higher degree of conservation than nucleotides in between these borders. Especially positions 1–6 can hardly be exchanged without severe loss of catalytic activity.

Each of the four guanosine residues were found to be highly conserved and cannot be exchanged by any of the other residues, i.e. cytidine, thymidine, or adenosine. DNA enzymes with cytidines at positions 3, 7, 10, or 13, substituted by adenosine, retained partial activity. This might be indicative of a functional role of the amino group at position 4 and 6 of the pyrimidine and purine ring system, respectively. Cytidine at position 7 can also be replaced by thymidine, and cytidine at position 10 by thymidine and guanosine without complete loss of activity. All adenosine residues except A8, which can only be replaced by cytidine, can be substituted by at least two other nucleotides without complete loss of catalytic function. Thymidine at position 4 is highly conserved, while thymidine at position 8 is the least conserved base within the catalytic center. The deoxyribozyme retains almost complete activity when T8 is exchanged by any of the other nucleotides.

Since only DNA enzymes with T, A, and C at position 8 were reported to show catalytic activity (12), we intended to check whether the observed activity of the DNAzyme with a guanosine in this position is specific for the sequence used. We therefore performed additional studies with a second deoxyribozyme, previously identified to cleave the VR1 mRNA efficiently. As can be determined from the gel shown in Fig. 4 (lane 2) the DNA enzyme 15 (9/9) cleaves ~87% of its target RNA when incubated with a 10-fold excess for 20 min at 37 °C. The activity of the mutated DNA enzyme (lane 3) did not differ significantly from the original DNAzyme indicating that a...
DNA enzyme with a T → G substitution at position 8 is highly active for different target sequences.

**Systematic Substitution of Core Nucleotides by Deoxynosine**—A systematic substitution of the bases by inosine was performed to further analyze the importance of amino and keto groups in the ring systems. Again, radioactively labeled target RNA was incubated with a 10-fold excess of the DNA enzymes for 20 min at 37 °C. Fig. 5 summarizes the results of the cleavage assays. As can already be seen in Fig. 5A, substitution of nucleotides at positions 3, 4, 5, 13, and 14 by inosine led to an almost complete loss of activity. We therefore intended to find out whether this amino group plays a functional role and analyzed the catalytic activity of a DNAzyme with deoxypurine at position 5. Low cleavage activity of this mutated DNAzyme compared with the unmodified DNA enzyme would confirm the importance of the amino group. Surprisingly, the mutated DNAzyme retained full cleavage activity (Fig. 6A).

To further analyze this finding, we performed kinetic experiments with the unmodified and mutated DNA enzyme and its 2600 base pairs long VR1 target mRNA. Despite the fact that a complete kinetic characterization is impossible with long target RNA molecules, we have recently shown that a 100-fold excess of the DNAzyme is sufficient to reach saturation. We should therefore be able to estimate the rate constant under these conditions. The $K_m$ value is of minor importance, since the substrate recognition arms remain unchanged. Target RNA degradation by the unmodified DNA enzyme and DNAzyme carrying an A→deoxypurine substitution is shown in Fig. 7. As can be seen in Table I, the observed reaction rate $k_{obs}$ of the mutated DNAzyme is even slightly higher than that of the unmodified DNA enzyme. We can therefore exclude any functional relevance of the amino group of the base at position 5.

Guanosine at position 14 had the highest degree of conservation in the catalytic center of the DNA enzyme. It could neither be exchanged by any of the naturally occurring nucleotides nor by inosine without almost complete loss of activity. Since inosine and guanosine differ only by the lack of the amino group at position 2 of the ring system, we have recently shown that a 100-fold excess of the DNA enzyme would confirm the importance of the amino group. Surprisingly, this mutated DNAzyme had no significant cleavage activity despite the presence of the amino group at position 2 of the ring system. We therefore conclude that both functional groups of guanosine 14 are essential for enzymatic activity.

**DISCUSSION**

The 10-23 DNA enzyme has been used for a variety of targets (1, 2) and has already successfully been applied in animal models (13–15). Our knowledge, however, about its mechanism and the importance of individual nucleotides in the catalytic domain is still limited. Here we describe a systematic study on the function of individual nucleotides in the catalytic domain of the 10-23 DNA enzyme.

We first replaced all of the core nucleotides separately by the other three naturally occurring nucleotides. Nucleotides at the borders of the catalytic center had a high degree of conservation. Especially positions G1, G2, T4, G6, and G14 could not be exchanged by any other nucleotide without complete loss of catalytic activity. This finding is in agreement with the original report on the selection of the 10-23 DNA enzyme where it was found to be almost completely intolerant of variation (12). Thymidine at position 8 was found to be the least conserved residue in accordance with earlier observations (12, 16). Replacement by adenine, guanosine, or cytidine had hardly any effect on cleavage activity.

Surprisingly, in contrast to the conserved borders of the core domain, nucleotides at positions 7–12 could be replaced by other naturally occurring nucleotides without severe effects. Only a C → G change at position 7 and an A → C change at
position 9 reduced cleavage of target RNA by more than 4-fold. Our data are consistent with a model for the 10-23 DNA enzyme that depicts the nucleotides at positions 1–6 and 13–14 to be directly involved in forming the catalytic center, as indicated in Fig. 1B.

To obtain further information on the importance of exocyclic functional groups, additional experiments were performed employing modified nucleotides. First, all nucleotides in the catalytic domain were replaced individually by inosine. Substitutions of nucleotides at positions 6, 8, 12, and 15 by inosine had only minor effects, while replacements of nucleotides at positions 3, 4, 5, 13, and 14 led to an almost complete loss of function.

The substitution of pyrimidines C3, T4, C7, T8, C10, and C13 by inosine had similar effects as their replacement by guanosine (Figs. 3 and 5). This finding can easily be explained by the close relationship between guanosine and inosine, which only lacks the exocyclic 2-amino group. The comparison of cytosine substitutions by adenosine and inosine reveals striking differences for positions 3 and 13. While the constructs with inosine are almost inactive, mutant DNA enzymes with adenosine at these positions maintain partial activity. Since the difference between the active adenosine constructs and the inactive inosine mutants is the exocyclic functionality, it is conceivable that the amino groups at position 3 or 13 may be of functional relevance.

Guanosine substitutions by inosine address the functional relevance of the exocyclic 2-amino group. While exchanges of G1 and G2 had moderate effects, no decrease in target cleavage was observed, when G6 was replaced by inosine. We thus conclude that the 2-amino group of G6 is not of functional importance. Since a G → A substitution at position 6 resulted in a loss of catalytic activity, it is conceivable that the 6-keto group plays an essential role in the catalytic event.

The replacement of guanosine at position 14 by inosine as well as by any of the other naturally occurring nucleotides led to an almost complete loss of function. This indicates that the 2-amino group of G14 is essential for cleavage. To further analyze whether this group is the only important exocyclic functionality we introduced 2-aminopurine that lacks the 6-keto group in comparison with guanosine. As can be seen in Fig. 6, this construct did not show any cleavage activity. Obviously, both functionalities of this essential guanosine at position 14 are important.

Compared with adenosine, the 6-amino function is replaced by a keto group in inosine. Replacement of adenosines at positions 9, 11, 12, and 15 had only moderate effects, excluding an essential role of their 6-amino groups. In contrast, introduction of inosine at position 5 had a significant effect on the catalytic activity. Combined with the observation that the construct...
with an A → C substitution at this position showed a reasonable activity, a potential role of the exocyclic amino group could be assumed. A replacement of adenosine at position 5 by deoxypurine, however, did not decrease the ability of the deoxyr-

bozyme to cleave its target RNA (Fig. 6). A closer kinetic analysis under saturating enzyme excess revealed even a slight increase of catalytic activity (Fig. 7 and Table I). We can thus exclude any functional relevance of the exocyclic keto group at position 5. It is conceivable that either the nitrogen in the ribose ring system is involved in hydrogen bond formation or the exocyclic keto groups of inosine, guanosine, and thymidine disturb the formation of an active conformer.

Our knowledge on the structure of the catalytic core of the 10-23 DNA enzyme is still very limited. NMR studies of the enzyme-substrate complex revealed base pairing of the substrate binding arms but could not resolve features of the catalytic core on the NMR time scale (16). In addition, two different high resolution structures have been published, but in both cases complexes are formed that are not relevant to catalytic function (18, 20). New crystals of the 10-23 DNA enzyme could be obtained by using a combinatorial screen of paired oligonucleotides (21), but the structure has not yet been published. Difficulties to obtain high resolution data of the catalytic domain may be related to our finding that the sequence in the catalytic core of the 10-23 DNA enzyme is surprisingly flexible. This variability may be related to a structural flexibility that is complicating NMR and crystallographic studies.

Once a high resolution structure of the DNA enzyme is known, our substitution analysis may provide complementary data for the interpretation of mechanistic details. As exemplified through the analysis of the hammerhead ribozyme, the importance of several positions with functional relevance could be confirmed by the crystal structure (22, 23). For some positions, however, the structural data were not in accordance with findings from substitution studies. While x-ray structures focus usually on the ground state, analog data also describe positions that are critical for the transition state. Differences between ground state structure based on x-ray crystal analysis and kinetic analyses using modified ribozymes are likely to reflect conformational changes upon formation of the transition state (9).

In summary, our substitution analysis of the 10-23 DNA enzyme revealed a high degree of flexibility at position 7–12 of the single-stranded region, whereas the highly conserved borders are likely to be directly involved in forming the catalytic site (Fig. 1B). A closer inspection of the functional relevance of exocyclic groups employing modified nucleotides excluded any importance of the amino groups of adenosine at position 5 and guanosine at position 6. The amino groups of cytidines at positions 3 and 13 and the keto group of guanosine at position 6 are likely to be involved in the formation of the active complex. In addition both functional groups of guanosine at position 14 were shown to be essential for target cleavage. Our results are likely to be useful for the functional interpretation of high resolution structures. In addition, these findings may help to design inactive control DNA enzymes and provide information about possible positions for the introduction of nucleotides with special functionality or modified nucleotides to protect the catalytic domain against endonucleolytic attack. Further work along this line is in progress.
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