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# LNAzymes: Incorporation of LNA-Type Monomers into DNAzymes Markedly Increases RNA Cleavage

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DNAzymes can function as specific RNA endonucleases by binding to predetermined sequences in an RNA and cleaving the phosphodiester backbone.[1] Highly efficient, sequence-specific cleavage of RNA is a prerequisite for the use of DNAzymes both as therapeutic antisense oligonucleotides and as general tools for manipulation of RNA. The most commonly used DNAzymes are derivatives of the 31-nucleotide "10-23" oligomer, which was originally isolated by in vitro selection.[1b] This DNAzyme attains its high specificity through hybridization of its two binding arms to complementary sequences immediately adjacent to the point of cleavage in the RNA substrate (Figure 1). Incorporation of phosphorothioate DNA or 2′-*O*-Me-RNA monomers into the binding arms provides resistance to exonucleolytic degradation without compromising RNA cleavage.[2] Between the binding arms in the middle of the DNAzyme is a 15-nucleotide sequence that constitutes the catalytic core.[1b]

We have previously reported the conformationally locked nucleic acid analogues LNA[3a,4] (locked nucleic acid) and the stereoisomeric α-L-LNA[3a,5] (α-L-configured LNA) (Figure 1). Both of these analogues possess a number of attractive features including substantially increased helical thermostability, excellent mismatch discrimination when hybridized with RNA (or DNA), and resistance toward exonucleolytic degradation.[4,5] In this report, we show that LNAzymes,[3b] that are designed to target a site in *Escherichia coli* 23S ribosomal RNA (rRNA), have markedly increased cleaving activity compared to that of a reference DNAzyme (Figure 1).

Oligonucleotides with two T nucleotides in each of the bindings arms replaced by α-L-LNA- or LNA thymine monomers (see Figure 1) were synthesized using published procedures.[4−6] We anticipated that this design would ensure increased substrate affinity while not preventing release of cleaved substrate. The cleaving efficiency was assessed by incubating each of the three enzymes with a $^{32}$P-labeled RNA transcript of 58 nucleotides (58n RNA),[7] which corresponds to a short stretch of the *E. coli* 23S rRNA sequence. After cleavage, products were separated by gel electrophoresis (Figure 2) and quantified by phosphor imager scanning. Single-turnover conditions with DNAzyme/LNAzyme:RNA ratios of 1:1, 10:1, and 50:1 showed that the LNAzymes **2** and **3** cleave much more avidly than the unmodified DNAzyme **1**. The DNAzyme only cleaved moderately well when present in a 50-fold molar excess, whereas

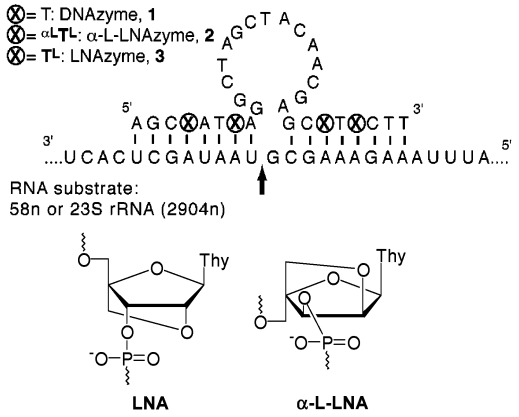


***Figure 1.*** Sequences of DNAzyme and LNAzymes[3b] and hybridization to the RNA target. Also shown are the structures of α-L-LNA ($^{\alpha L}$**T**$^{L}$) and LNA (**T**$^{L}$) thymine nucleotide monomers (Thy = thymin-1-yl). The arrow points at the cleavage site.

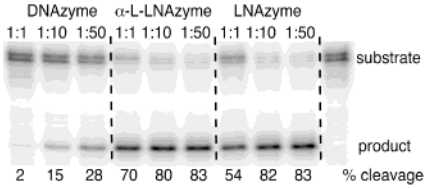


***Figure 2.*** Denaturing gel electrophoresis after cleavage (60-min incubation) of the 58n RNA under single-turnover conditions.[7] "1:1", "1:10", and "1:50" denote substrate to DNAzyme/LNAzyme ratios used. See Supporting Information for explanation of double-substrate band.

stoichiometric amounts of the two LNAzymes were sufficient to cleave >50% of the RNA substrate.

A more detailed analysis, performed over a range of reaction times (Figure 3), confirmed that the LNAzymes **2** and **3** were significantly more efficient than the DNAzyme **1** at cleaving the RNA. As the LNA nucleotides are incorporated in the binding arms, the improvement is probably due to a higher hybridization affinity of the LNAzymes for the RNA substrate. This explanation is supported by bandshift analysis of the hybridization complexes run on nondenaturing polyacrylamide gels and by similar experiments performed with a less-structured 17n RNA substrate having a sequence identical to the hybridizing region of the 58n substrate (see Supporting Information for results and details). It has been reported that cleavage activity of a DNAzyme was reduced by approximately 50-fold after its binding arms had been substituted with RNA sequences.[8] However, under single-turnover conditions there does not seem to be a significant difference between the effects

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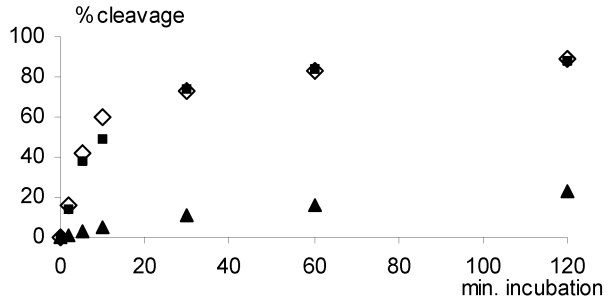


***Figure 3.*** Time-dependent cleavage of the 58n RNA.[7] (■) α-L-LNAzyme; (◇) LNAzyme; (▲) DNAzyme. The DNAzyme:substrate ratio was 50:1, while LNAzyme:substrate ratios were 5:1.

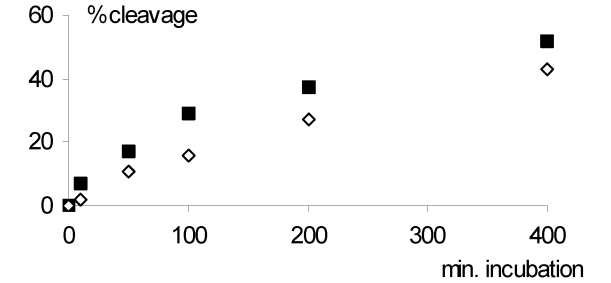


***Figure 4.*** Curves showing multiple-turnover cleavage of the 58n RNA.[7] (■) α-L-LNAzyme; (◇) LNAzyme. The substrate:LNAzyme ratio was 20:1.

of binding arms containing either α-L-LNA or LNA nucleotides. This is despite the fact that the LNA binding arms would take on a more RNA-like, A-form helix conformation.[9,10]

Under multiple-turnover conditions both LNAzymes display efficient multiple turnover (Figure 4). These preliminary results suggest the α-L-LNAzyme to be somewhat more efficient than the LNAzyme which could be linked to the rate of product release. The unmodified DNAzyme exhibited no measurable turnover (<2% cleavage, not shown) under these conditions. Similar experiments were performed with the less-structured 17n substrate, confirming not only the results depicted in Figure 4 but also the importance of the secondary structure of the RNA substrate as the DNAzyme induced 5% cleavage (400-min incubation; DNAzyme-to-substrate ratio = 1:20) in this case (see Supporting Information for results and details).

We additionally evaluated how the LNAzymes cleave their target under single-turnover conditions in a mixture of 5S, 16S, and 23S rRNAs (120n, 1542n, and 2904n) extracted from *E. coli* cells. Primer extension with reverse transcriptase generates gel bands corresponding to 23S rRNA cleaved at the target nucleotide 1093, as well as to the uncut rRNA (Figure 5).[11] Cleavage is equivalent to that seen for the 58n substrate with both the LNAzymes displaying considerably improved performance compared to the DNAzyme.

In conclusion, we have shown that incorporation of α-L-LNA and LNA nucleotides into the binding arms of a DNAzyme markedly improves the efficiency of RNA cleavage. Furthermore, both the LNAzymes exhibit efficient turnover. These LNAzymes are able to cleave efficiently the phosphodiester backbone at the target nucleotide not only in a small RNA substrate but also in a

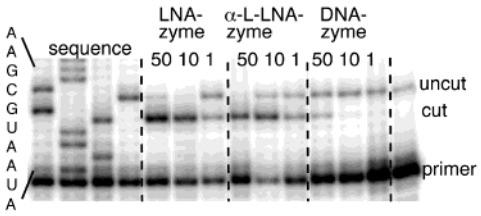


***Figure 5.*** Denaturing gel electrophoresis of primer extension analyses of 23S rRNA after cleavage (45-min incubation) by the DNAzyme and LNAzymes.[7,11] "50", "10", and "1" denote substrate to DNAzyme/LNAzyme ratios of 1:50, 1:10, and 1:1, respectively.

naturally occurring ribosomal RNA at a highly structured target region. The results reported here show that compared to a DNAzyme, LNAzymes gain significantly improved access to an RNA target, as well as distinctly increase the rate of RNA cleavage.

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**Supporting Information Available:** Preparation of the 58n transcript and the ribosomal RNA, details of the cleavage reactions, gel-shift analysis, and results for the 17n RNA substrate (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

## References

(1) (a) Breaker, R. R.; Joyce, G. F. *Chem. Biol.* **1994**, *1*, 223. (b) Santoro, S. W.; Joyce, G. F. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 4262.
(2) (a) Warashina, M.; Kuwabara, T.; Nakamatsu, Y.; Taira, K. *Chem. Biol.* **1999**, *6*, 237. (b) Sioud, M.; Leirdal, M. *J. Mol. Biol.* **2000**, *296*, 937. (c) Liu, C.; Cheng, R.; Sun, L. Q.; Tien, P. *Biochem. Biophys. Res. Commun.* **2001**, *284*, 1077. (d) Cieslak, M.; Niewiarowska, J.; Nawrot, M.; Koziolkiewicz, M.; Stec, W. J.; Cierniewski, C. S. *J. Biol. Chem.* **2002**, *277*, 6779.
(3) (a) We define LNA as an oligonucleotide containing one or more 2′-*O*,4′-*C*-methylene-β-D-ribofuranosyl nucleotide monomer(s) (ref 4a). Similarly, we define α-L-LNA as an oligonucleotide containing one or more 2′-*O*,4′-*C*-methylene-α-L-ribofuranosyl nucleotide monomer(s) (ref 5a). (b) We define an LNAzyme as a DNAzyme containing one or more LNA-type nucleotide monomer(s).
(4) (a) Singh, S. K.; Nielsen, P.; Koshkin, A. A.; Wengel, J. *Chem. Commun.* **1998**, 455. (b) Koshkin, A. A.; Singh, S. K.; Nielsen, P.; Rajwanshi, V. K.; Kumar, R.; Meldgaard, M.; Olsen, C. E.; Wengel, J. *Tetrahedron* **1998**, *54*, 3607. (c) Obika, S.; Nanbu, D.; Hari, Y.; Andoh, J.; Morio, K.; Doi, T.; Imanishi, T. *Tetrahedron Lett.* **1998**, *39*, 5401. (d) Koshkin, A. A.; Nielsen, P.; Meldgaard, M.; Rajwanshi, V. K.; Singh, S. K.; Wengel, J. *J. Am. Chem. Soc.* **1998**, *120*, 13252. Wengel, J. *Acc. Chem. Res.* **1999**, *32*, 301.
(5) (a) Rajwanshi, V. K.; Håkansson, A. E.; Dahl, B. M.; Wengel, J. *Chem. Commun.* **1999**, 1395. (b) Sørensen, M. D.; Kværnø, L.; Bryld, T.; Håkansson, A. E.; Verbeure, B.; Gaubert, G.; Herdewijn, P.; Wengel, J. *J. Am. Chem. Soc.* **2002**, *124*, 2164.
(6) The purity of the LNAzymes **2** and **3** was verified (>90%) by capillary gel electrophoresis and the composition by MALDI-MS ($[M + H]^+$ *m/z* (found/calcd): **2**, 9696/9598; **3**, 9597/9598).
(7) The following buffer was used for the cleavage reactions: 10 mM $MgCl_2$, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 37 °C. See Supporting Information for further details.
(8) Ota, N.; Warashine, M.; Hirano, K.; Hatanaka, K.; Taira, K. *Nucleic Acids Res.* **1998**, *26*, 3385.
(9) Petersen, M.; Håkansson, A. E.; Wengel, J.; Jacobsen, J. P. *J. Am. Chem. Soc.* **2001**, *123*, 7431.
(10) Petersen, M.; Bondensgaard, K.; Wengel, J.; Jacobsen, J. P. *J. Am. Chem. Soc.* **2002**, *124*, 5974.
(11) Sigmund, C. D.; Ettayebi, M.; Borden, A.; Morgan, E. A. *Methods Enzymol.* **1988**, *164*, 673.

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