Synthesis and NMR Studies of the RNA-Cleaving DNA Enzyme

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Single-stranded DNA can fold into well-defined, sequence-dependent tertiary structures, specifically bind to a variety of target molecules and exhibit catalytic activities similar to those of ribozymes or protein enzymes.[1] Their high chemical stability, amenability to the introduction of chemical modifications and low cost of synthesis make DNA enzymes attractive as therapeutic agents and designed catalysts. Catalytic DNA enzymes were isolated in the laboratory by *in vitro* selection from a large pool of random oligonucleotides and that were found to have a variety of activities, including RNA cleavage reaction, ligation of chemically activated DNA and incorporation of metal atom into porphyrin.[2,3] Of particular interest were deoxyribozymes with RNA-cleaving activity for which consists of a 19-nucleotide oligomer containing a single ribonucleotide as a substrate and a 38-deoxyribonucleotide oligomer as a catalyst.[4,5] This enzyme is composed of a catalytic core flanked by two substrate-recognition domains that bind the substrate through Watson-Crick base pairing (Figure 1). Moreover, this substrate has a short stretch of an unpaired substrate nucleotide, 5'-GGA-3', between the two regions involved in the base pairing, which is important in substrate recognition, metal coordination, or other aspects of catalytic function.[5]

To understand clearly and in more detail the mechanism of how the DNA enzyme works, knowledge of the three-dimensional structure is essential. We synthesized these oligomers in sufficient amounts, and proton Nuclear Magnetic Resonance spectra of the complex were measured. The system studied consisted of a non-cleavable DNA substrate bound to a DNA enzyme. Since a divalent ion, such as magnesium, is required for efficient catalytic activity of the DNAzyme, the NMR data were also obtained as a function of the Mg^{2+} concentration.

The single-stranded oligonucleotides, d(GGAAGAGATG), d(CATCTCTGAA) d(CACTATAGGAAGAGATG) and d (CATCTCTGAAGTAGCGCCGCCGTATAGTG) were synthesized by the phosphoramidite method on a DNA synthesizer (Applied Biosystem, model 391). The crude 5-dimethoxytritilated oligonucleotides were deprotected by treatment with concentrated ammonia for 12 hours at 55 °C. The DNA products were purified by reverse-phase High Performance Liquid Chromatography and desalted by Sephadex G-25 column. The duplex was prepared by dissolving the substrate (17 mer) and DNAzyme (29 mer) oligonucleotide strand (adjusted to a stoichiometric 1 : 1 ratio) in an aqueous solution containing 10 mM sodium phosphate and 100 mM NaCl while monitoring one-dimensional ^{1}H NMR. Hydroxyapatite DNA grade column with 50-400 mM phosphate buffer (pH 6.8) gradient was used to isolate the duplex among the single strands. The duplex was desalted by Sephadex G-25 column. All NMR experiments were carried out using Bruker DMX-600 spectrometers. The data were processed using a Silicon Graphics workstation with the program *XWIN* and *FELIX 95.0*.

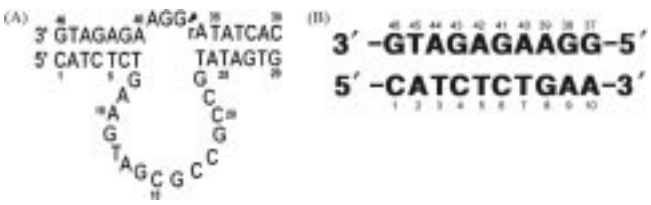

Figure 1. (A) Secondary structure of the complex between the DNAzyme (29 mer) and substrate (17 mer). The substrate contains a single adenosine ribonucleotide, flanked by deoxyribonucleotidese. (B) DNA sequence contexts of the left stem of the complex.

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Proton NMR spectra of an equimolar mixture of the enzyme and substrate were measured in H_2O (Figure 2A). Hydrogen bonded imino proton resonances are usually observed in the 12-15 ppm region and unpaired imino proton resonances for a hairpin are observed in the 9.5-12 ppm region. The complex shows signals in both the regions, suggesting that it contains base-paired stems and unpaired loops in which the imino protons are in a hydrophobic environment. Figure 2B shows the titration of the complex with 0 to 19 mM of added Mg^{2+}. There are no big chemical shift changes as a whole, but there are a slight downfield shift and broadening of the resonances with increasing Mg^{2+} concentration in the 12-15 ppm region. These NMR data show that the binding of the magnesium ion does not induce a significant conformational change in this system. Therefore, it is inferred that having magnesium ions bound loosely to the complex has no effect on the structure but on the function. To assign the resonances for the stems, proton NMR spectra of the left stem were measured (Figure 1B).

All imino protons were assigned following the standard analysis scheme of labile protons.[6] The imino protons of all G·C base pairs were assigned by observing the Nuclear Overhauser Effects from the C amino protons, which were assigned by intraresidue NOEs of H5 ↔ NH2, to the G imino proton of the opposed base. The imino protons of all T·A base pairs were assigned from NOEs between the T imino protons and the H2 protons of the opposite A residue (Figure 3A). The left stem is assumed to adopt a typical B-form-like structure from the absence of the discontinuities in the sequential NOEs of imino to imino protons. However, the significantly weaker intensities for T5-NH ↔ G43-NH and G43-NH ↔ T3-NH, compared with those of the other NOEs, indicate that these base pairs are destabilized. Several NOE cross peaks were assigned

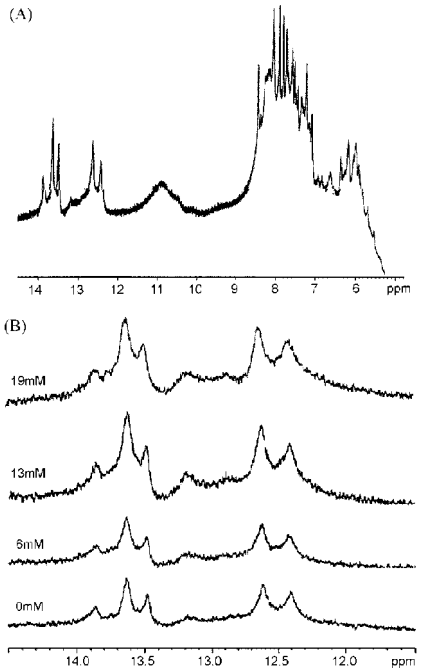

Figure 2. (A) ^{1}H NMR spectra for the DNAzyme-substrate complex in H_2O buffer solution containing 10 mM sodium phosphate (pH 7.0) and 100 mM NaCl. (B) Mg^{2+}-dependence of the imino proton resonances of the ^{1}H NMR spectra for the complex in H_2O buffer solution. The Mg^{2+} concentrations are shown on the left.

tentatively to the chemical exchange of T5, G41 and T7 bases. NOEs for T7-NH ↔G8-NH, G38-NH ↔G8-NH and G37-NH ↔G38NH were not observed. This suggests that stacking interaction is absent in this region of the left stem.

The non-exchangeable proton assignments for the left stem were based on the sequential assignment procedure. First, cytosine base proton resonances were identified by their H5 and H6 cross peaks, followed by the assignment of base and sugar proton resonances.[6] Sequential NOE connectivities between the base H6/H8 and sugar H1' protons were well observed (Figure 3B). The base proton assignments were confirmed by observing the NOEs from the base to H2'/H2" resonances. The chemical shifts and assignments for the proton resonances of the left stem are listed in Table 1.

Table 1. Chemical shifts of proton resonances in the left stem

Base	NH	NH2/H2	H6/H8	H1'	H5/CH_3
C1		8.28	7.62	5.54	
A2		7.64	8.32	6.21	
T3	13.39		7.13	5.78	1.28
C4		8.00/6.75	7.46	5.89	5.40
T5	13.48		7.31	5.94	1.42
C6		8.15/6.90	7.38	5.85	5.41
T7	13.73		7.03	5.80	1.37
G8					
A9					
A10					
G37					
G38					
A39					
A40		7.15	7.59	5.58	
G41	12.32		8.12	5.22	
A42		7.39	7.90	5.92	
G43	12.54		7.51	6.39	
A44		7.68	7.92	6.39	
T45	13.08		6.95	5.73	
G46	12.70				

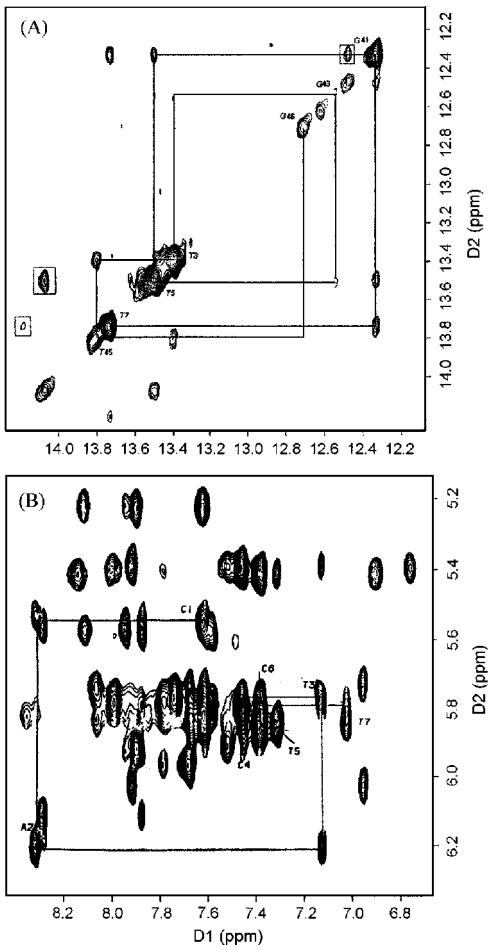

Figure 3. Expanded NOESY contour plots of the left stem. (A) Sequential connectivity between imino protons of the (n)- and (n+1)-base pairs. And chemical exchange peaks are shown in rectangle. (B) A typical region (base to H1' protons) in the sequential connectivity of the DNAzyme strand.

Using proton NMR spectroscopy, we have demonstrated the secondary structural features of the DNA enzyme complex consisting of a DNA enzyme bound to a non-cleavable DNA substrate. A full report on the NMR studies of structure determination of the RNA-cleaving DNA enzyme will be published in the future.

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