

# NMR Exchange Study of Z-DNA Duplexes Induced by the Z $\alpha$ Domain of Human ADAR1 Containing Mutation on the $\beta$ -Hairpin

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Left-handed Z-DNA forms in polymers of alternating d(CG)<sub>n</sub> sequence and is a higher energy conformation than B-DNA.<sup>1,2</sup> Z-DNA is stabilized by high salt, negative supercoiling, and complex formation with Z-DNA binding proteins.<sup>1-3</sup> The human double-stranded RNA deaminase I (ADAR1) contains two Z-DNA binding domains (Z $\alpha$  and Z $\beta$ ) at the NH<sub>2</sub>-terminus.<sup>4,5</sup> The crystal structure of the Z $\alpha$  domain of human ADAR1 (Z $\alpha$ <sub>ADAR1</sub>) complexed to Z-DNA showed that each of two Z $\alpha$  domains binds to one strand of a double-stranded DNA, yielding two-fold symmetry with respect to the DNA helical axis.<sup>6</sup> This study also revealed that the intermolecular interaction is mediated by 5 residues in the  $\alpha$ 3 helix and 4 residues in the  $\beta$ -hairpin (Fig. 1).<sup>6</sup> This

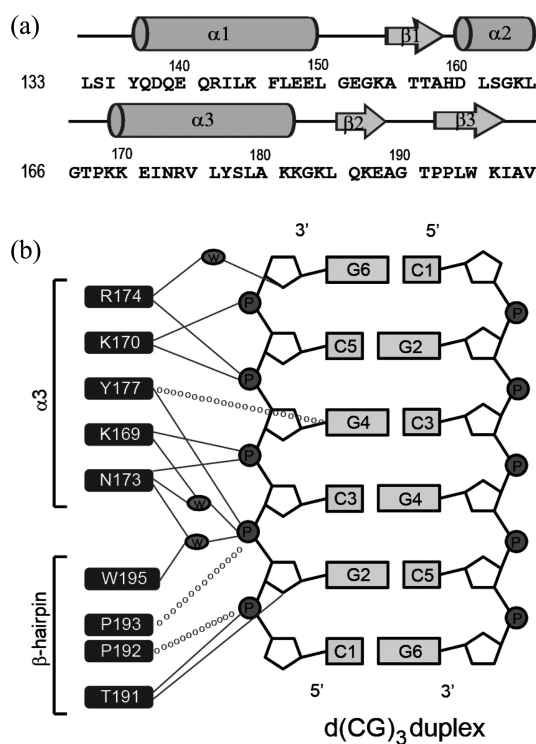
is confirmed in solution by the significant backbone chemical shift changes of most residues in the  $\alpha$ 3 helix and  $\beta$ 2-loop- $\beta$ 3 region upon binding to Z-DNA.<sup>7</sup> These residues play important roles in Z $\alpha$  function and thus replacement of them with an alanine caused a dramatic decrease in Z-DNA binding affinity compared to wild-type Z $\alpha$ <sub>ADAR1</sub> (wt-Z $\alpha$ <sub>ADAR1</sub>).<sup>5</sup> Previous NMR study suggested that single mutations at residues K169, N173, or Y177 cause unusual conformational changes in the hydrophobic faces of helices  $\alpha$ 1,  $\alpha$ 2, and  $\alpha$ 3, which dramatically decrease the Z-DNA binding affinity.<sup>8</sup> This study also found that the K170A and R174A mutants significantly destabilize the G2·C5 base pair compared to wt-Z $\alpha$ <sub>ADAR1</sub>, even though these mutants could efficiently change B-DNA to left-handed Z-DNA.<sup>8</sup> However, the question of how the mutants on the  $\beta$ -hairpin affect the base pair stabilities of the Z-DNA remains.

Here, we determined hydrogen exchange rate constants ( $k_{ex}$ ) for the imino protons of the d(CG)<sub>3</sub> in the complexes with Z $\alpha$ <sub>ADAR1</sub> containing mutation on the  $\beta$ -hairpin. Comparison of these results to previous studies of the mutant Z $\alpha$ <sub>ADAR1</sub>-d(CG)<sub>3</sub> complexes<sup>8</sup> provides the information required to identify the role of each residue in the  $\beta$ -hairpin during B-Z transition of DNA duplexes.

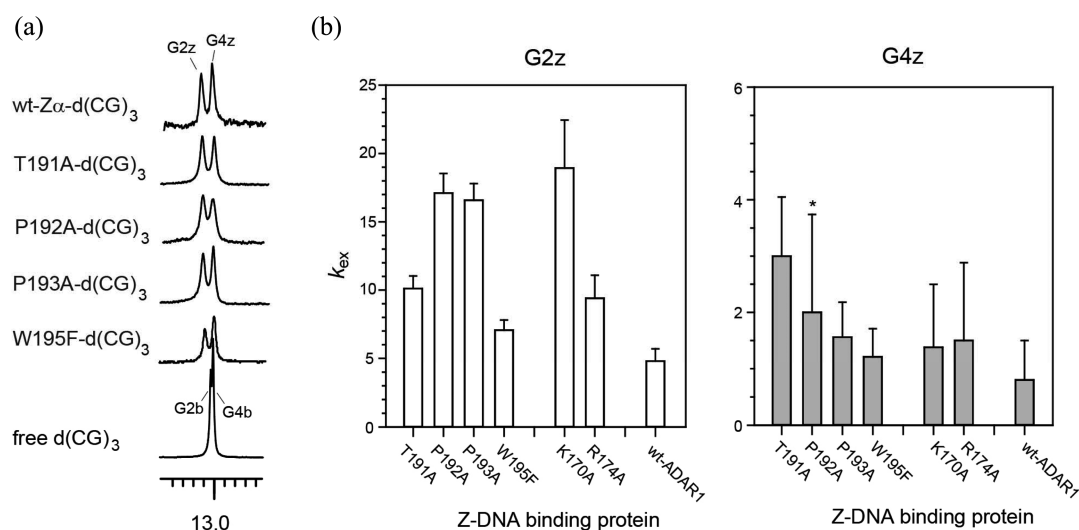
## Experimental Section

All DNA oligonucleotides were purchased from M-bio-tech Co. (Seoul, Korea). The oligonucleotides were purified by reverse-phase HPLC and desalted by Sephadex G-25 column. The coding sequence DNAs for site-directed mutagenesis were purchased from BIONEER Inc. (Korea) and were cloned into an *E. coli* expression vector pET28a (Novagen, WI, USA). To produce <sup>15</sup>N-labeled mutant Z $\alpha$ <sub>ADAR1</sub>, BL21(DE3) bacteria were grown in M9 medium that contained 1 g/L <sup>15</sup>NH<sub>4</sub>Cl. The <sup>15</sup>N-labeled mutant Z $\alpha$ <sub>ADAR1</sub> proteins were expressed and purified as described elsewhere.<sup>9</sup> The DNA and protein samples were dissolved in a 90% H<sub>2</sub>O/10% D<sub>2</sub>O NMR buffer containing 10 mM sodium phosphate (pH 8.0) and 10 mM NaCl.

NMR experiments were carried out on a Agilent DD2 700 MHz spectrophotometer (GNU, Jinju) equipped with x, y, z-axis pulsed-field gradient cold probe. NMR data were processed and analyzed with the program FELIX2004 (FELIX



**Figure 1.** (a) Sequence of the Z $\alpha$ <sub>ADAR1</sub> proteins. Numbering and secondary structure elements for Z $\alpha$ <sub>ADAR1</sub> are shown on top of the sequence. (b) Residues of Z $\alpha$ <sub>ADAR1</sub> involved in interaction with the d(CG)<sub>3</sub> DNA duplex reported in a previous study.<sup>6</sup> Intermolecular hydrogen bonds are indicated by solid lines and van der Waals contacts are shown by open circles.



**Figure 2.** (a) 1D imino proton spectra of free d(CG)<sub>3</sub> and d(CG)<sub>3</sub> in complexes with T191A (P/N = 3.0), P192A (P/N = 2.5), P193A (P/N = 2.5), and W195F (P/N = 3.5) mutant proteins at 35 °C. (b) Exchange rate constants ( $k_{ex}$ ) of the G2z and G4z imino protons for the T191A-d(CG)<sub>3</sub>, P192A-d(CG)<sub>3</sub>, P193A-d(CG)<sub>3</sub>, and W195F-d(CG)<sub>3</sub> complexes at 35 °C. The  $k_{ex}$  values for the G2z and G4z imino protons in the complexes with K170A,<sup>8</sup> R174A,<sup>8</sup> and wt-Zα<sub>ADAR1</sub><sup>7</sup> are shown on the right. Error bars represent curve fitting errors during the determination of  $k_{ex}$  from water magnetization transfer data. The asterisk symbol (\*) indicates that the  $k_{ex}$  value of this imino proton could not be determined exactly because of precipitation during NMR experiment.

NMR, CA) or VNMRJ (Agilent, CA). The exchange rates of the imino protons were determined as previously described.<sup>10</sup> The hydrogen exchange rates of the imino protons were measured by water magnetization transfer experiments.<sup>10</sup> The imino hydrogen exchange rate constants ( $k_{ex}$ ) were determined by fitting the data to Eq. (1):<sup>10</sup>

$$\frac{I(t)}{I_0} = 1 - 2 \frac{k_{ex}}{(R_{1w} - R_{1a})} (e^{-R_{1a}t} - e^{-R_{1w}t}) \quad (1)$$

where  $R_{1a}$  and  $R_{1w}$  were the independently measured and are the apparent longitudinal relaxation rates of the imino proton and water, respectively, and  $I_0$  and  $I(t)$  are the peak intensities of the imino proton in the water magnetization transfer experiments at times zero and  $t$ , respectively.<sup>10</sup>

## Results and Discussion

The imino proton resonance assignment of the d(CG)<sub>3</sub> in complex with mutant Zα<sub>ADAR1</sub> proteins were assigned by comparing the NOESY spectra with those of the wt-Zα<sub>ADAR1</sub>-d(CG)<sub>3</sub> complex. Figure 2(a) shows the imino proton spectra of d(CG)<sub>3</sub> in complexes with Zα<sub>ADAR1</sub> containing site-specific T191A, P192A, P193A, or W195F mutations. The new resonances (G2z and G4z) are indicative of the Z-form helix generation. In T191A, P192A, and P193A mutants, most of the d(CG)<sub>3</sub> duplexes were converted to Z-DNA at P/N ratios ≥ 2.0, like wt-Zα<sub>ADAR1</sub> (Fig. 2(a)). These data mean that the mutations at residues T191, P192, or P193 have little effect on the B-Z transition activities of Zα<sub>ADAR1</sub> proteins. In W195F, about 95% of the d(CG)<sub>3</sub> duplexes displayed the Z-form when the P/N ratio increased up to 3.5 (Fig. 2(a)), indicating that the W195F mutant has slightly lower B-Z transition activity than wt-Zα<sub>ADAR1</sub>.

In order to compare the conformational stabilities of the Z-DNA duplexes induced by mutant Zα<sub>ADAR1</sub> proteins, the hydrogen exchange rate constants ( $k_{ex}$ ) of the imino protons in the T191A-d(CG)<sub>3</sub>, P192A-d(CG)<sub>3</sub>, P193A-d(CG)<sub>3</sub>, and W195F-d(CG)<sub>3</sub> complexes were determined at 35 °C by the water magnetization transfer method.<sup>7,10</sup>

The  $k_{ex}$  data of the imino protons in the complexes were obtained by curve fitting the intensity data to Eq. (1). Figure 2(b) shows the  $k_{ex}$  data for the G2z and G4z imino protons in the T191A-d(CG)<sub>3</sub>, P192A-d(CG)<sub>3</sub>, P193A-d(CG)<sub>3</sub>, and W195F-d(CG)<sub>3</sub> complexes at 35 °C, where the majority of the d(CG)<sub>3</sub> duplexes exhibited the Z-conformation. The G2z imino protons in the complexes with T191A ( $10.1 \pm 0.9$  s<sup>-1</sup>) and W195F ( $7.1 \pm 0.7$  s<sup>-1</sup>) have 2.1- and 1.5-fold larger  $k_{ex}$  values, respectively, compared to wt-Zα<sub>ADAR1</sub> (Fig. 2(b)). These results indicate that the loss of hydrogen bonding interaction between the side-chains of residues 191 or 195 and the DNA phosphate backbone in the Zα<sub>ADAR1</sub>-d(CG)<sub>3</sub> complex caused destabilization of the G2-C5 base pair. Interestingly, the  $k_{ex}$  values of G2z in the complexes with P192A ( $17.1 \pm 1.4$  s<sup>-1</sup>) and P193A ( $16.6 \pm 1.2$  s<sup>-1</sup>) are significantly larger than those of the wt-Zα<sub>ADAR1</sub>-d(CG)<sub>3</sub> as well as T191A-d(CG)<sub>3</sub> and W195F-d(CG)<sub>3</sub> complexes (Fig. 2(b)), meaning that the hydrophobic interaction between the P192 or P193 side-chains and the DNA backbone contributes to the stabilization of the G2-C5 base pair with more efficiency than hydrogen bonding interaction of the T191 and W195 side-chains.

Similar to K170A and R174A, the G4z imino protons in the complexes with P192A, P193A, and W195F have  $k_{ex}$  values in the range of 1.2–2.0 s<sup>-1</sup> (Fig. 2(b)), which is slightly larger than that of wt-Zα<sub>ADAR1</sub>-d(CG)<sub>3</sub> complex.<sup>7</sup> Surprisingly, in the T191A-d(CG)<sub>3</sub> complex, the  $k_{ex}$  value of G4z

( $3.0 \pm 1.0 \text{ s}^{-1}$ ) is 4-fold larger than that of the wt- $Z\alpha_{\text{ADARI}}$ -d(CG)<sub>3</sub> complex (Fig. 2(b)). This result implicates that the hydrogen bonding interaction between T191 side-chain and the C1pG2 phosphate lead to stabilization of the G2·C5 base-pair and as well as stabilization of neighboring C3·G4 base-pair in Z-DNA in contrast to residues P192, P193, and W915. This is consistent with previous CD data where the two neighboring residues (190 and 191) of the conserved P191-P192 site play important roles in binding to and stabilization of Z-DNA by a  $Z\alpha$  protein.<sup>11</sup>

The crystal structure of the  $Z\alpha_{\text{ADARI}}$ -d(CG)<sub>3</sub> complex revealed that five residues (K169, K170, N173, R174, and Y177) in the  $\alpha 3$  helix form direct or water-mediated hydrogen bonds with backbone phosphates in one strand of the d(CG)<sub>3</sub> duplex (Fig. 1(b)).<sup>6</sup> Among these residues, residues K169, N173 and Y177 show a high degree of conservation among various Z-DNA binding proteins and play important roles in  $Z\alpha$  function. It was reported that N173A displayed the most dramatic decrease in Z-DNA binding with 160-fold lower affinity than wt- $Z\alpha_{\text{ADARI}}$ .<sup>5</sup> The CD experiments revealed that the K169A and Y177F mutants showed significantly lower B-Z transition activities.<sup>12</sup> Hydrogen exchange study suggested that the hydrogen bonding interaction between K170 and R174 side-chains and the phosphate backbone plays an important role in the base-pair stabilization in Z-DNA rather than in the active B-Z transition pathway.<sup>8</sup>

Recent NMR studies with the  $Z\alpha_{\text{ADARI}}$ -d(CG)<sub>3</sub> complex have suggested an active B-Z transition mechanism, in which (i) one molecule of  $Z\alpha_{\text{ADARI}}$  (P) preferentially binds to B-DNA (B) and forms a B-DNA- $Z\alpha_{\text{ADARI}}$  (BP) complex; (ii) then the B-form helix of the BP complex is converted to left-handed Z-DNA (ZP complex); (iii) finally, the stable Z-DNA-( $Z\alpha_{\text{ADARI}}$ )<sub>2</sub> (ZP<sub>2</sub>) complex is produced by addition of a second P to a ZP complex.<sup>7</sup> Previous study suggested that the K170A and R174A proteins could efficiently convert the B-form helix of the d(CG)<sub>3</sub> duplex to left-handed Z-DNA via an active B-Z transition pathway like wt- $Z\alpha_{\text{ADARI}}$  protein.<sup>8</sup> Like these two proteins, the mutants  $Z\alpha_{\text{ADARI}}$ , which contain mutations on the  $\beta$ -hairpin, also could efficiently changes the B-DNA to left-handed Z-DNA.

In the  $Z\alpha_{\text{ADARI}}$ -d(CG)<sub>3</sub> complex, residues T191 and W195 in the  $\beta$ -hairpin exhibit direct or water-mediated hydrogen bonding interactions with phosphate backbone or bases of the d(CG)<sub>3</sub> duplex (Fig. 1(b)).<sup>6</sup> In addition, residues P192, P193, and W195 participate in hydrophobic interactions with phosphate backbone or bases of the d(CG)<sub>3</sub> duplex (Fig. 1(b)).<sup>6</sup> Our hydrogen exchange data suggests that both the hydrogen bonding and hydrophobic interactions of the  $\beta$ -hairpin with Z-DNA play important roles in the stabilization of the G2·C5 base-pair. It was reported that the P192A and P193A mutations of  $Z\alpha_{\text{ADARI}}$ -fused E3L significantly decrease lethality compared to wild-type virus.<sup>12</sup> This result suggests that the stabilization of Z-DNA induced by a  $Z\alpha$  protein may play an important roles in poxviral infection. Especially, the hydrogen bonding interactions of the T191 side-chain with

the C1pG2 phosphate and sugar oxygen of the G2 residue contribute to the stabilization of the G2·C5 as well as C3·G4 base-pairs in Z-DNA. Recently, the CD studies on the various mutants  $Z\alpha_{\text{ADARI}}$  reported that the T191K mutant did not affect B-Z transition activity of  $Z\alpha_{\text{ADARI}}$  protein but has 2-fold larger  $k_{\text{off}}$  rate than wt- $Z\alpha_{\text{ADARI}}$ .<sup>13</sup> This means that this greater stabilities of Z-DNA base-pairs induced by inter-molecular interaction of T191 side-chain contributes to stabilization of the bound form of  $Z\alpha_{\text{ADARI}}$ -Z-DNA complex rather than destabilization of the unbound form.

In summary, we determined the  $k_{\text{ex}}$  values of the imino protons in the d(CG)<sub>3</sub> in complexes with  $Z\alpha_{\text{ADARI}}$  containing mutation on the  $\beta$ -hairpin (T191A, P192A, P193A, and W195F). The 1D imino proton spectra showed that alanine substitution at residues T191, P192, P193, or W195 slightly affected the Z-DNA binding affinity, but these residues did not play crucial roles in the B-Z transition. A hydrogen exchange study proved that the loss of hydrogen bonding or hydrophobic interactions between the side-chains of residues 191, 192, 193 or 195 and the DNA phosphate backbone in the  $Z\alpha_{\text{ADARI}}$ -d(CG)<sub>3</sub> complex caused significant destabilization of the G2·C5 as well as C3·G4 base pairs.

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