NMR study of the Z-DNA binding mode and B–Z transition activity of the Zα domain of human ADAR1 when perturbed by mutation on the α3 helix and β-hairpin

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The Zα domains of human ADAR1 (Z\( _{\text{ADAR1}} \)) bind to Z-DNA via interaction mediated by the α3-core and β-hairpin. Five residues in the α3 helix and four residues in the β-hairpin play important roles in Zα function, forming direct or water-mediated hydrogen bonds with DNA backbone phosphates or interacting hydrophobically with DNA bases. To understand the roles of these residues during B–Z transition of duplex DNA, we performed NMR experiments on complexes of various Z\( _{\text{ADAR1}} \) mutants with a 6-bp DNA duplex at various protein-to-DNA molar ratios. Our study suggests that single mutations at residues K169, N173, or Y177 cause unusual conformational changes in the hydrophobic faces of helices α1, α2, and α3, which dramatically decrease the Z-DNA binding affinity. A hydrogen exchange study proved that the K170A- and R174A-Z\( _{\text{ADAR1}} \) proteins could efficiently change B-DNA to left-handed Z-DNA via an active B–Z transition pathway, whereas the G2 C5 base pair was significantly destabilized compared to wild-type Z\( _{\text{ADAR1}} \).

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**Introduction**

Left-handed Z-DNA, which is a polymer of alternating d(CG)\( _{n} \) sequence, is a higher energy conformation than B-DNA [1,2]. Z-DNA is induced by high salt, negative supercoiling, and complex formation with Z-DNA binding proteins [1–3]. Z-DNA binding domains are found in the RNA editing enzyme (ADAR1)\(^{2}\) and DNA-dependent activator of IFN-regulatory factor (DAI) in vertebrates, the E3L protein of poxviruses, and the fish PKR-like protein kinase (PKZ). The human ADAR1 protein contains two tandem Z-DNA binding domains (Z\( \alpha \) and Z\( \beta \)) at the NH\( _{2} \)-terminus [4,5].

The crystal structure of the Z\( \alpha \) domain of human ADAR1 (Z\( _{\text{ADAR1}} \)) in complex with Z-DNA showed that each of two Z\( \alpha \) domains binds to one strand of a double-stranded DNA, yielding twofold symmetry with respect to the DNA helical axis [6]. The crystal structure of the Z\( \beta \) domain of human ADAR1 (Z\( _{\text{ADAR1}} \)) reported that Z\( _{\text{ADAR1}} \) maintains a winged helix-turn-helix (HTH) fold along with a C-terminal helix (α4) [7]. The Z\( \alpha \) domains of the yatapoxviral E3L (yabZ\( _{\text{E3L}} \)) and mouse DAI (mZ\( _{\text{DAI}} \), also known as DLM-1 or ZBP1) show sequence homology to Z\( _{\text{ADAR1}} \) (Fig. 1A) and their overall structures and interactions with Z-DNA are similar to Z\( _{\text{ADAR1}} \) [8,9]. Structural studies with the domain of human DAI (Z\( _{\text{DAI}} \)) in complex with Z-DNA suggest that Z\( _{\text{DAI}} \) has a structure that is similar to other Z-DNA binding proteins, but adopts an unusual conformation when binding to Z-DNA [10].

Although these structural studies explain well the specificities of Z\( \alpha \) family proteins for Z-DNA, it remains unclear how Z\( \alpha \) protein specifically recognizes Z-DNA sequence in a sea of B-DNA to produce the stable Z\( \alpha \)-Z-DNA complex during the B–Z transition induced by Z\( \alpha \). Recent NMR studies of the complex formed between Z\( _{\text{ADAR1}} \) and a 6-base-paired (6-bp) DNA duplex (referred to as d(CG)\( _{n} \), Fig. 1B) have suggested an active B–Z transition mechanism, in which the Z\( _{\text{ADAR1}} \) protein first binds to B-DNA and then converts it to left-handed Z-DNA, a conformation that is subsequently stabilized by the additional binding of a second Z\( _{\text{ADAR1}} \) molecule [11]. It was also reported that yabZ\( _{\text{E3L}} \) can efficiently

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\( ^{1} \) These two authors contributed equally to this work.

\( ^{2} \) Abbreviations used: ADAR1, RNA editing enzyme; DAI, DNA-dependent activator of IFN-regulatory factor; PKZ, PKR-like protein kinase; Z\( _{\text{ADAR1}} \), Z\( \alpha \) domain of human ADAR1; Z\( _{\text{DAI}} \), Z\( \beta \) domain of human ADAR1; HTH, helix-turn-helix; yabZ\( _{\text{E3L}} \), Z\( \alpha \) domain of the yatapoxviral E3L; mZ\( _{\text{DAI}} \), Z\( \alpha \) domain of the mouse DAI; Z\( _{\text{DAI}} \), Z\( \beta \) domain of human DAI; wt, wild-type P/N ratio; protein-to-DNA ratio; \( \kappa_{\text{ex}} \), hydrogen exchange rate constant; 2D, two-dimensional; orfZ\( _{\text{E3L}} \), Z\( \alpha \) domain of the orf virus E3L.
change the 6-bp B-form helix to left-handed Z-DNA via an active B–Z transition pathway [12]. Recently, Hohng et al. suggested a conformational selection mechanism in which intrinsic Z-DNAs are dynamically formed and effectively stabilized by Zx proteins through efficient trapping of the Z conformation [13].

The crystal structure of the d(CG)3–ZADAR1 complex [6] revealed that the interaction is mediated by 5 residues in the α3 core and 4 residues in the β-hairpin (Fig. 1B). This is confirmed in solution by the significant backbone chemical shift changes of most residues in the α3 helix and β2-loops of the region upon binding to Z-DNA [11]. Among these residues, K169, N173, Y177, and W195 show a high degree of conservation among various other Z-DNA binding proteins (Fig. 1A). These residues play important roles in Zx function and thus replacement of them with an alanine caused a dramatic decrease in Z-DNA binding affinity compared to wild-type ZADAR1 (wt-ZADAR1) [5]. Even though biochemical studies on the DNA binding affinities and B–Z transition activities of various mutant Z-DNA binding proteins have been performed systematically [5,9,14–16], the detailed molecular mechanism of how these mutations affect the B–Z transition pathway of DNA duplexes with different activities is still not understood.

Here, we performed NMR experiments on complexes of several ZADAR1 mutants with the d(CG)3 DNA duplex (Fig. 1B) at various protein-to-DNA (P/N) molar ratios. We investigated the conformational changes induced in ZADAR1 by single site mutations in the α3 helix as well as the β-hairpin using NMR spectroscopy. In the mutant ZADAR1 proteins, the chemical shift perturbations in the amide backbone upon binding to Z-DNA were also studied. The hydrogen exchange rate constants \( k_{ex} \) for the imino protons of the d(CG)3 in the complexes with mutant ZADAR1 were also determined as a function of the P/N ratios. Comparison of this results to previous studies of the ZADAR1–d(CG)3 complex [11] provides the information required to identify the role of each residue in the α3 helix and the β-hairpin during B–Z transition of DNA duplexes.

Materials and methods

Sample preparation

The DNA oligomer, d(CG)3, was purchased from M-biotech Inc. (the Korean branch of IDT Inc.), purified by reverse-phase HPLC, and desalted using a Sephadex G-25 gel filtration column. The coding sequence DNAs for site-directed mutagenesis were purchased from BIONEER Inc. (Korea) and were cloned into Escherichia coli expression vector pET28a with an N-terminal His-tag (Novagen, WI, USA), and the expression vectors were used to transform E. coli strain BL21(DE3). Uniformly 15N-labeled ZADAR1 mutants were obtained by growing the transformed Escherichia coli cells in M9 medium that contained 1 g/L 15NH4Cl. The 15N-labeled mutant ZADAR1 proteins were purified with a Ni–NTA affinity column and a Sephacyr S-100 gel filtration column (GE Healthcare, USA) on a GE AKTA Prime Plus as described elsewhere [17]. The DNA and protein samples were dissolved in a 90% H2O/10% D2O NMR buffer containing 10 mM sodium phosphate (pH 8.0) and 100 mM NaCl.

NMR Experiments

All of the 1H and 15N NMR experiments were performed on an Agilent DD2 700-MHz spectrometer (GNN) equipped with a cold probe. All two- and three-dimensional (2D and 3D) NMR experiments were carried out with 0.5 mM 15N-labeled wild-type or mutant ZADAR1 proteins. The imino proton and 1H/15N-HSQC spectra were obtained for complexes prepared by addition of 15N-labeled proteins to 0.2 mM DNA samples in NMR buffer at the indicated P/N ratio. 1D NMR data were processed with either VNMRFIT (Agilent, CA, USA) or Felix 2004 (FELIX NMR, CA, USA) software, while the 2D and 3D data were processed with NMRPIPE [18] and analyzed with Sparky [19]. External 2,2-dimethyl-2-silapentane-5-sulfonate was used for the 1H and 15N references.

1H and 15N backbone resonance assignment for wt-ZADAR1 was previously reported [11] and resonances for mutant ZADAR1 proteins were assigned by comparing their 3D NOESY-1H/15N-HSQC spectra with that of the wt-ZADAR1. The imino proton resonance assignments for free d(CG)3 and the d(CG)3–ZADAR1 complex were as previously reported [11]. The average chemical shift differences of the amide proton and nitrogen resonances between the free ZADAR1 and ZADAR1 in complex with d(CG)3 were calculated by using Eq. (1):

\[ \Delta \delta_{\text{avg}} = \sqrt{\left(\Delta \delta_{\text{p}}\right)^2 + \left(\Delta \delta_{\text{n}}/5.88\right)^2} \]

where \( \Delta \delta_{\text{p}} \) and \( \Delta \delta_{\text{n}} \) are the chemical shift differences of the amide proton and nitrogen resonances, respectively.
Hydrogen exchange rate measurement

Hydrogen exchange rate constants of the imino protons of d(CG₃) in complex with mutant ZₓADAR₁ at various P/N ratios were determined by a water magnetization transfer experiment. The intensities of each imino proton were measured with 20 different delay times. The kₑₓ values for the imino protons were determined by fitting the data to Eq. (2):

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

(2)

where I₀ and I(t) are the peak intensities of the imino proton at times zero and t, respectively, and R₁a and R₁w are the apparent longitudinal relaxation rate constants for the imino proton and water, respectively; these parameters were measured with semi-selective inversion recovery 1D NMR experiments [20,21].

Results

Assignments of amide backbone resonances of mutant ZₓADAR₁ proteins

NMR studies on the K169A-, K170A-, N173A-, R174A-, Y177I, T191A-, P192A-, P193A-, and W195F-ZₓADAR₁ proteins were performed to compare the effect of mutations in the α3 helix and β-hairpin on the DNA binding and B–Z transition activity. The amide backbone resonances of the mutant ZₓADAR₁ proteins were assigned by comparing their 3D NOESY-¹H/¹⁵N-HSQC or 2D ¹H/¹³⁵N-HSQC spectra with those of the wt-ZₓADAR₁. The secondary structures of all mutant ZₓADAR₁ proteins were assessed from sequential and medium-range NH–NH and NH-Hz NOE patterns in their NOESY-¹H/¹⁵N-HSQC spectra (Supplementary Fig. S1). The α₁-β₁-α₂-β₂-β₃ secondary structure of the wt-ZₓADAR₁ [5,6,22] was basically unchanged in all ZₓADAR₁ mutants, allowing the use of chemical shift mapping to probe the Z-DNA binding site (Supplementary Fig. S1).

The superimposed ¹H/¹⁵N-HSQC spectra for wild-type and mutant ZₓADAR₁ proteins at 25 °C are shown in Supplementary Figs. S2–S10. As shown in Fig. 2, mutation at residues K169, K170, N173, R174, or Y177 caused significant chemical shift changes in several residues of the α3 helix, which are located close to the mutated residue. In K169A-ZₓADAR₁, the K169 mutation causes chemical shift changes >0.05 ppm in the amide resonances of residues 170, 172, and 174–177 (Fig. 2A). In the K170A-ZₓADAR₁ mutant, residues 171 and 172 presented backbone chemical shift changes of 0.26 and 0.07, respectively (Fig. 2B). In N173A-ZₓADAR₁, chemical shift changes of >0.05 ppm were observed in residues 171, 174–178, and 180–181 (Fig. 2C). In R174A-ZₓADAR₁ mutant, the mutation caused chemical shift changes >0.05 ppm in the amide resonances of residues 169–171, 173, and 175–176 (Fig. 2D). In Y177I-ZₓADAR₁, residues 174–176 and 178–181 underwent significant chemical shift changes >0.05 ppm upon replacement of Y177 with an isoleucine (Fig. 2E). This mutation also caused significant chemical shift changes of two N173-H residues (0.37 ppm) (Supplementary Fig. S11), indicating that the side-chains of both residues are closely located to each other in the ZₓADAR₁ structure (Fig. 2E).

In T191A-ZₓADAR₁, no residues underwent chemical shift changes >0.05 ppm (Fig. 2F and Supplementary Fig. S7). In P192A-ZₓADAR₁, the mutation caused chemical shift changes >0.05 ppm in the amide resonances of residues 187–191 and 194–196 of the β2-loop–β3 region and in A158 of the α2 helix (Fig. 2G). Similarly, in P193A-ZₓADAR₁, residues 198, 190–191, and 194–195 of the β2-loop–β3 region and A158 of the α2 helix underwent chemical shift changes >0.05 ppm (Fig. 2H).

Both P192A and P193A mutations caused significant chemical shift changes in the W195-Hs: resonance (Fig. 2G and H and Supplementary Fig. S11).

In W195F-ZₓADAR₁, some residues of the α3 helix (173, 175–183) and the β2 (184–187 and 189) and β3 sheets (194 and 196–199) underwent significant chemical shift changes larger than 0.05 ppm upon replacement of W195 with a phenylalanine (Supplementary Fig. S11). Similar to Y177I-ZₓADAR₁, the W195F mutation caused chemical shift changes in the two N173-Hs2 resonances (0.12 and 0.17 ppm) (Supplementary Fig. S11).

Surprisingly, in N173A-ZₓADAR₁, residues 157, 160, 162–163 and 165–166 in the α2-loop region presented significant backbone chemical shift changes, even though these residues are far from residue 173 (Fig. 2C). Similarly, in K169A-ZₓADAR₁, chemical shift changes >0.05 ppm were observed in residues 160–162 and 164–167 in the α2-loop region (Fig. 2A). In Y177I-ZₓADAR₁, significant chemical shift changes were observed in residues S162 and L165 (Fig. 2E). Similar results were also observed in residues of the β2-loop–α2 region (152–155, 157–160, 162–164, and 166–167) in W195F-ZₓADAR₁ (Fig. 2I and Supplementary Fig. S11).

Titrations of mutant ZₓADAR₁ proteins to d(CG₃) duplex

The imino proton resonance assignment of the free d(CG₃) DNA duplex was previously reported [11] and the resonances of the d(CG₃) in complex with mutant ZₓADAR₁ proteins were assigned by comparing the NOESY spectra with those of the wt-ZₓADAR₁-d(CG₃) complex (see Supplementary Fig. S12). Fig. 3 shows the change in the imino proton spectra of d(CG₃) upon titration with the mutant ZₓADAR₁ proteins. The new resonances (G2z and G4z) are indicative of the Z-form helix generation. However, the chemical shifts of these G imino resonances are slightly different from each other (Supplementary Table S1). In the presence of K169A-ZₓADAR₁, the G2z resonance (13.10 ppm) exhibits slight up-field shifts compared to the wt-ZₓADAR₁–d(CG₃) complex (13.15 ppm). This NMR data indicates that, in solution, the G2-C5 base-pair geometry in the Z-DNA helices can be affected by removal of the hydrogen bonding interaction between K169’s side-chain and the phosphate backbone of the DNA. Interestingly, in the K170A-ZₓADAR₁–d(CG₃) and R174A-ZₓADAR₁–d(CG₃) complexes, the G4z imino resonances have different chemical shifts compared to the Z-DNA induced by wt-ZₓADAR₁ (Supplementary Table S1), indicating that intermolecular interaction of the side-chains of K170 and R174 residues are important for the C3-G4 base-pair geometry, as well as the G2-C5 base-pair, in the Z-DNA helices induced by ZₓADAR₁ proteins.

In K169A-ZₓADAR₁, only about 25% of the d(CG₃) duplexes displayed the Z-conformation at P/N = 2.0 (Fig. 3B), whereas most of the d(CG₃) duplexes were converted to Z-DNA by wt-ZₓADAR₁ at a P/N ratio ≥2.0 [11]. At P/N = 9.0, a majority of the d(CG₃) exhibited the Z-conformation (Fig. 3B). These results indicate that the K169A-ZₓADAR₁ protein has at least 4 times lower B–Z transition activity than wt-ZₓADAR₁. The K170A-ZₓADAR₁ and R174A-ZₓADAR₁ proteins had significantly higher B–Z transition activities compared to K169A-ZₓADAR₁ (Fig. 3C and E). At P/N = 2.0, about 60% and 85% of the d(CG₃) duplexes displayed the Z-conformation in the complexes with K170A-ZₓADAR₁ and R174A-ZₓADAR₁, respectively (Fig. 3C and E). In T191A-, P192A-, and P193A-ZₓADAR₁, most of the d(CG₃) duplexes were converted to Z-DNA at P/N ratios ≥2.0, like wt-ZₓADAR₁ (Fig. 3G–I). These data mean that the mutations at residues T191, P192, or P193 have little effect on the B–Z transition activities of ZₓADAR₁ proteins. In W195F-ZₓADAR₁, about 50% and 95% of the d(CG₃) duplexes displayed the Z-conformation at P/N = 2.0 and 3.5, respectively (Fig. 3J), indicating that the W195F mutant has 2-fold lower B–Z transition activity than wt-ZₓADAR₁.
proteins, no significant chemical shift changes were observed in the spectra of the complex with N173A-ZADAR1 proteins: red, >0.20 ppm; magenta, 0.10–0.20 ppm; gold, 0.05–0.10 ppm. Coordinates for ZADAR1 were derived from the X-ray crystal structure (PDB id = 1BJ)[6]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In Y177I-ZADAR1, a new resonance appeared at 13.18 ppm with very weak peak intensity at P/N = 2.0 (denoted as x in Fig. 3F). However, it was not confirmed that this resonance is indicative of Z-form helix generation, because the peak intensity of this resonance did not increase even though the P/N ratio increased up to 15.0 (Fig. 3F). Similarly, a new imino proton resonance was also observed in the spectra of the complex with N173A-ZADAR1 (P/N = 4.0) (Fig. 3D) but characterization of this resonance could not be performed because the samples for free N173A-ZADAR1 and the N173A-ZADAR1-d(CG)3 complex precipitated during NMR experiments.

Chemical shift changes in mutant ZADAR1 proteins upon d(CG)3 binding

A superposition of the 1H/15N-HSQC spectra for free mutant ZADAR1 proteins and the ZADAR1 mutants bound to d(CG)3 at 35 °C is shown in the Supplementary data (Figs. S13 and S21). The weighted average 1H/15N backbone chemical shift changes were determined for each residue using Eq. 1 (Fig. 4). The chemical shift perturbation results for T191A-, P192A-, and P193A-ZADAR1 upon binding to d(CG)3 were very similar to those reported previously for wt-ZADAR1[11] (Fig. 4). Similar results were also observed in K170A-, R174A-, and W195F-ZADAR1, even though their chemical shift changes were relatively smaller than those of wt-ZADAR1 (Fig. 4). In contrast to these six mutant ZADAR1 proteins, the residues of K169A-ZADAR1 underwent chemical shift changes <0.10 ppm upon binding to d(CG)3 (Fig. 4). In the N173A- and Y177I-ZADAR1 proteins, no significant chemical shift changes occurred upon exposure to the d(CG)3 duplex (Fig. 4D and F). This result means that N173A- and Y177I-ZADAR1 have very low binding affinities to the d(CG)3 duplex compared to wt- and the other six mutant ZADAR1 proteins and that they exist primarily in the free state rather than the d(CG)3-bounded form.

In K169A-ZADAR1, most residues in the α3 helix underwent chemical shift changes of 0.05–0.10 ppm upon binding to d(CG)3 (Fig. 4B), whereas in wt-ZADAR1, all residues in the α3 helix exhibited chemical shift changes >0.1 ppm [11]. Residues in the α2 and β3 regions showed very small chemical shift changes (<0.05 ppm), unlike wt-ZADAR1 (Figs. 4B and 5). In addition, small or no chemical shift changes were observed in the β2-loop-β3 region (see T191 in Fig. 5B and I197 in Fig. 5F).

In the K170A-ZADAR1-d(CG)3 complex, most residues of the α3 helix exhibited chemical shift changes larger than 0.10 ppm (Fig. 4C). Interestingly, the amide resonances for four residues (173, 174, 176, and 177) of the α3 helix disappeared altogether (Fig. 4C and Supplementary Fig. S14), indicating that there is chemical exchange between free and DNA-bound forms of K170A-ZADAR1 on an intermediate NMR time scale. In addition, significant chemical shift changes were observed in the β1-α2 and β2-loop-β3
regions (Fig. 4C). However, these resonances, such as A158, T167, and K196, exhibited significantly less movement upon binding to d(CG)$_3$ than the same residues of wt-Z$_a$ADAR1 (Fig. 5).

In the R174A-Z$_a$ADAR1–d(CG)$_3$ complex, most residues of the $\alpha$3 helix as well as residues in the $\beta$1-$\alpha$2 and $\beta$2-loop-$\beta$3 regions exhibited significant chemical shift changes, similar to wt-Z$_a$ADAR1 (Fig. 4E). Similar to K170A-Z$_a$ADAR1, the amide resonances for residues R174, L176, and Y177 of the $\alpha$3 helix disappeared altogether upon binding to d(CG)$_3$ (Fig. 4E and Supplementary Fig. S16).

In the T191A, P192A-, and P193A-Z$_a$ADAR1–d(CG)$_3$ complex, most residues of the $\alpha$3 helix exhibited chemical shift changes larger than 0.10 ppm (Fig. 4G–I). In addition, most residues of the $\beta$1-$\alpha$2 and $\beta$2-loop-$\beta$3 regions exhibited significant chemical shift changes, although the perturbation results were slightly different from wt-Z$_a$ADAR1 (Fig. 4G–I).

In the W195F-Z$_a$ADAR1–d(CG)$_3$ complex, most residues of the $\alpha$3 helix as well as residues in the $\beta$2-loop-$\beta$3 region exhibited significant chemical shift changes, similar to wt-Z$_a$ADAR1 (Fig. 4C). Similar to K170A- and R174A-Z$_a$ADAR1, the amide resonances for residues R174, L176, and Y177 of the $\alpha$3 helix disappeared altogether upon binding to d(CG)$_3$ (Fig. 4J and Supplementary Fig. S21). Interestingly, residues A158, S162, and L165 in the $\beta$1-$\alpha$2 region presented chemical shift changes >0.1 ppm, whereas other residues in the $\beta$1-$\alpha$2 region showed relatively smaller chemical shift changes compared to wt-Z$_a$ADAR1 (Fig. 4J and Supplementary Fig. S21).

Exchange rate constants of the d(CG)$_3$ imino protons in the complexes with K170A- and R174A-Z$_a$ADAR1

In order to compare the conformational stabilities of the Z-DNA duplexes induced by mutant Z$_a$ADAR1 proteins, the hydrogen exchange rate constants ($k_{ex}$) of the imino protons in the K170A-Z$_a$ADAR1–d(CG)$_3$ and R174A-Z$_a$ADAR1–d(CG)$_3$ complexes were determined at 35 °C by the water magnetization transfer method [11,20]. The $k_{ex}$ data of the imino protons in the complexes were obtained by curve fitting the intensity data to Eq. 2. In the K170A-Z$_a$ADAR1–d(CG)$_3$ complex, no significant changes in the $k_{ex}$ data for the G4b imino protons between P/N = 1.0 and P/N = 2.0 were observed (data not shown). Similar results were also observed

![Fig. 3. 1D imino proton spectra of the d(CG)$_3$ duplex at 35 °C upon titration with (A) wt- (B) K169A- (C) K170A- (D) N173A- (E) R174A- (F) Y177I- (G) T191A- (H) P192A- (I) P193A- and (J) W195F-Z$_a$ADAR1. The resonances from B-form DNA were denoted as G2b and G4b and those from Z-form were denoted as G2z and G4z. P/N ratios are shown on the left of each spectrum.](image-url)
in the kex data for the G2b and G4b imino protons in the R174A-ZADAR1–d(CG)3 complex. These results indicated that the binding of d(CG)3 to K170A-ZADAR1 or R174A-ZADAR1 had little effect on the kex data for the G2b and G4b imino protons.

Fig. 6 shows the kex data for the G2z and G4z imino protons in the K170A-ZADAR1–d(CG)3 and R174A-ZADAR1–d(CG)3 complexes at various P/N ratios. In the K170A-ZADAR1–d(CG)3 complex, the kex value for the G2z imino proton decreased from 38.8 to 19.0 s⁻¹ as the P/N ratio increased from 1.0 to 3.0 (Fig. 6). Similarly, the G2z imino proton of the R174A-ZADAR1 showed significant decrement of the kex value from 35.7 to 9.5 s⁻¹, as the P/N ratio increased (Fig. 6). Similar results were also observed in the kex data for the G2z imino proton of the wt-ZADAR1–d(CG)3 complex [11] as well as the yabZ-E3L–d(CG)3 complex [12]. This observation can be explained by the presence of a mixture of G2 imino resonances from two complex states, the ZADAR1–d(CG)3 and (ZADAR1)₂–d(CG)3 complexes, in the G2z peak. Similarly, the kex value for the G4z imino protons in the both complexes also decreased as the P/N ratio increased (Fig. 6). At P/N = 3.0, where the majority of the d(CG)3 duplexes exhibited the Z-conformation, the G2z imino protons in the K170A-ZADAR1–d(CG)3 and R174A-ZADAR1–d(CG)3 complexes have the kex values of 19.0 ± 3.5 s⁻¹ and 9.5 ± 1.7 s⁻¹, respectively (Fig. 6). Surprisingly, these kex values are significantly larger than those of the wt-ZADAR1–d(CG)3 and yabZ-E3L–d(CG)3 complexes, whereas there are no clear differences in the kex value of G4z imino proton between these complexes (Fig. 6). These results indicate that the loss of hydrogen bonding interaction between the side-chains of residues 170 or 174 and the DNA phosphate backbone in the ZADAR1–d(CG)3 complex caused significant destabilization of the G2/C1/C5 base pair but had little effect on the conformational stability of the C3/C1/G4 base pair.

Discussion

The Zα domains of various Z-DNA binding proteins bind to Z-DNA via interactions mediated by 5 residues in the α3 helix and 4 residues in the β-hairpin (Figs. 1B and 7A) [6,8,9,23]. A previous NMR study [11] found that most residues in the α3 helix and β2-loop–β3 region underwent backbone chemical shift changes larger than 0.1 ppm upon binding to the d(CG)3 substrate (Fig. 7B), indicating the direct interaction of their side chains with the phosphate backbone as reported in the previous crystal structural study [6]. Interestingly, some residues in the β1–α2 region also underwent
protein requires backbone conformational change during complex formation with Z-DNA to achieve its full Z-DNA binding affinity and B–Z transition activity. The crystal structure of the d(CG)₃–ZₐADAR₁ complex revealed that five residues (K169, K170, N173, R174, and Y177) in the α3 helix form direct or water-mediated hydrogen bonds with backbone phosphates in one strand of the d(CG)₃ duplex (Figs. 1B and 7A). Among these residues, residues K169, N173 and Y177 show a high degree of conservation among various ZₐADAR₁ as well as other kinds of Z-DNA binding proteins (see Fig. 1A), suggesting that they play important roles in Zₐ function. It was reported that the N173A mutant displayed the most dramatic decrease in Z-DNA binding of all α3 mutations, with 160-fold lower affinity than wild-type ZₐADAR₁ [5]. This is consistent with our NMR results showing that there are no clear changes in the imino proton spectra (Fig. 3D) or the ¹H/¹⁵N HSQC spectra (Supplementary Fig. S15) during titration of d(CG)₃ with N173A-ZₐADAR₁.

It was reported that the Y177A mutant had significantly lower Z-DNA binding affinity (26-fold reduction), relative to wt-ZₐADAR₁ [5,15]. In addition to hydrogen bonding interaction via a hydroxyl group, Y177 is likely to participate in CH…π stacking with the G4 nucleotide (Figs. 1B and 7A). Mutants Y177K [5] and Y177F [15], which are capable of either hydrogen bonding or hydrophobic interactions, respectively, could bind better to Z-DNA than Y177A, but still worse than wt-ZₐADAR₁. This suggests that the side-chain of Y177 does not play a crucial role for the B–Z conformational change of DNA, although it partially contributes to Z-DNA binding through hydrophobic and/or hydrogen bonding interactions. Surprisingly, this study found that Y177L-ZₐADAR₁ could not induce significant changes in the imino proton spectra of the d(CG)₃, even up to a P/N ratio of 15.0 (Fig. 3F). It is reported that the ZₐADAR₁ protein, which consists of I335 at the corresponding position 177 strongly interferes with not only DNA binding but also the B–Z conformational change of DNA via induction of an unusual backbone conformational change, even though this residue can form hydrophobic interactions with the DNA bases.

Recent NMR studies with the ZₐADAR₁–d(CG)₃ complex have suggested an active B–Z transition mechanism, in which (i) one molecule of ZₐADAR₁ (P) preferentially binds to B-DNA (B) and forms a B-DNA–ZₐADAR₁ (BP) complex with K_{BP}^{PP} = [B][P]/[BP] × 10⁻⁷ M; (ii) then the B-form helix of the BP complex is converted into the Z-DNA helix.
Fig. 7. (A) Surface model of wt-ZADAR1 for representing the intermolecular interaction with one strand of Z-DNA. (B) Surface model of 1H15N chemical shift changes (Δδavg) in ZADAR1 upon d(CG)3 binding, as previously reported [11]. Colors used to illustrate Δδavg are red, >0.20 ppm; magenta, 0.10–0.20 ppm; and gold, 0.05–0.10 ppm. Coordinates for ZADAR1 bound to Z-DNA were derived from the X-ray crystal structure (PDB id = 1QBJ) [6]. The gray color indicates the hydrophobic residues essential for correct folding of ZADAR1. (C) A comparative stick view of the d(CG)3 duplex in complex with ZADAR1 (PDB id = 1QBJ) [6]. The green lines indicate direct or water-mediated hydrogen bonds of K169, K170, and R174 with backbone phosphates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

At the left-handed Z-DNA (the ZP complex) with K_{DZ} = [ZP]/[BP] ~ 1; (iii) finally, the stable Z-DNA-(ZADAR1)2 (ZP2) complex is produced by addition of a second P to a ZP complex with K_{DZP2} = [ZP][P]/[ZP2] ~ 87K_{BP} [11]. When the total protein concentration is high enough to promote almost full Z-DNA binding in the ZP2 conformation, the apparent dissociation constant (K_{DPP} = [B][P]/[ZP2]) can be used to compare the B–Z transition activities of the ZADAR1 mutants with wild-type protein. A previous study reported that mutant K169A showed significant loss of Z-DNA binding affinity (37-fold reduction) [5]. However, we observed significant changes in both the imino proton spectra of d(CG)3 (Fig. 3A) and the 1H15N HSQC spectra (Fig. 5) of K169A-ZADAR1 upon binding, although the B–Z transition activity was worse than with wt-, K170A-, and R174A-ZADAR1. As described in the Supplementary data, the K_{DPP} value of the K169A-ZADAR1 could be calculated by the equation K_{DPP} = [P][1 - f_2]/f_2, where f_2 is the Z-DNA fraction, because a majority of the d(CG)3 exhibited the Z-conformation at P/N ratios >9.0 (Fig. 3A). When it is assumed that the NMR sample at P/N = 9.0 was prepared by a mixture of protein (total concentration: 0.9 mM) and DNA (total concentration: 0.1 mM), the final concentration of free K169A-ZADAR1 is ~0.7 mM. If the Z-DNA population is approximately 98% at P/N = 9.0, the K_{DPP} value of the K169A-ZADAR1 is calculated as ~1 × 10^{-8} M^{-1}, which is significantly larger than that of wt-ZADAR1 (~1 × 10^{-12} M^{-2}). This calculation suggests why significant changes in this 1D imino and 1H15N HSQC spectra are observed, even though this mutant has about 37-fold lower Z-DNA binding affinity than the wild-type protein, as reported in the previous study [5].

Based on this active B–Z transition mechanism, the k_{ex} value for the G2z imino proton depends on the relative concentrations of the ZP and ZP2 complexes as a function of the P/N ratio [11]. This study of the d(CG)3 duplexes complexed with K170A- and R174A-ZADAR1 showed that the k_{ex} values for the G2z and G4z imino protons are significantly decreased as the P/N ratio is increased (Fig. 6). This result strongly suggests that the K170A- and R174A-ZADAR1 proteins could efficiently convert the B-form helix of the d(CG)3 duplex to left-handed Z-DNA via an active B–Z transition pathway like wt-ZADAR1 protein. Surprisingly, the G2z imino protons in the complexes with K170A- and R174A-ZADAR1 have 4- and 2-fold larger k_{ex} values, respectively, compared to wt-ZADAR1 (Fig. 6), indicating that the hydrogen bonding interaction between K170 and R174 side-chains and the phosphate backbone plays an important role in the stabilization of the G2-C5 base-pair in Z-DNA rather than in the active B–Z transition pathway.

1D imino proton and 1H15N-HSQC spectra showed that the K170A- and R174A-ZADAR1 proteins have significantly higher B–Z transition activities compared to the other three ZADAR1 mutants, but still lower affinities than wt-ZADAR1 (Figs. 3 and 5). Southwestern assays in previous studies showed that mutants K170A and R174A had only a minor effect on Z-DNA binding [5]. Although the yabZesk protein has a glutamine at the corresponding site of K170 (see Fig. 1A), it exhibits the same B–Z transition activity as wt-ZADAR1 [16]. The Zesk domain from orf virus (orfZesk), which has a histidine at the corresponding site of K170 (see Fig. 1A), shows less Z-DNA binding affinity than ZADAR1 and yabZesk [16]. We interpret this to mean that mutations at residues K170 and/or R174 in the ZADAR1 protein slightly affect its Z-DNA binding affinity, but they do not play crucial roles in the B–Z transition of DNA duplexes.

Although our NMR results on the various mutant ZADAR1 proteins were consistent with previous results of band shift and Southwestern assays, BiACore experiments and CD spectra [5,9,14–16], the detailed molecular mechanism of how these mutants affect the B–Z transition pathway of DNA duplexes with different activities is not well understood. Substitution of residues K170, R174, or T191 with an alanine caused chemical shift perturbation in several residues whose amide protons are located very close to their side-chains (Fig. 2). This suggests that K170, R174, and T191 contribute to Z-DNA binding via only hydrogen bonding interactions with the phosphate backbone. In addition, P192 and P193 contribute to Z-DNA binding through hydrophobic interac-
tions with the DNA backbone. In K169A-, N173A-, and Y177-ZnADAR1, some residues located near to the mutated residue exhibit significant chemical shift changes like K170- and R174-ZnADAR1, but in these three mutants, significant chemical shift changes were also observed in the L165 residue of the α2-loop region, although these residues are far from the mutated site (Fig. 2). It was suggested that the hydrophobic faces of helices α1 (I143 and L144), α2 (L161and L165), and α3 (I172, V175, and L179) contribute to a hydrophobic core essential for correct folding of ZnADAR1, similar to that found in other HTH-motif proteins [5]. Thus, conformational changes in the α2-loop region as well as the α3-loop and β2-loop regions are required in order for ZnADAR1 proteins to form complex with Z-DNA (Fig. 7C). This can explain why most residues of the α2 helix showed significant chemical shift perturbation upon binding to the d(CG)3 substrate, even in the absence of any direct interaction with Z-DNA (Fig. 7B). Our NMR results revealed that mutations at residues K169, N173, or Y177 cause unusual backbone conformational changes in the α2-loop region, which can propagate to the α3 helix via hydrophobic interaction among helices α1, α2, and α3. This induced conformational change in the α3 helix might affect hydrogen bonding interactions between its side-chains and the phosphate backbone, abrogating efficient binding to Z-DNA. Thus, it is concluded that the dramatic decrement of Z-DNA binding affinity in the K169A-, N173A-, and Y177I-ZnADAR1 mutants is mainly caused by disruption of the hydrogen bonding interactions between the side-chains of the α3 helix and the DNA phosphate backbone, induced by conformational change in the hydrophobic faces rather than removal of their side-chains.

The crystal structure of the ZnADAR1–d(CG)3 complex [6] revealed that the side-chains of K169, R174, and K170 in ZnADAR1 formed hydrogen bonds with three phosphate anions of the C3pG4pC5pG6 steps, respectively, in one strand of the d(CG)3 duplex (Fig. 7D). The ZnADAR1 protein displayed a different hydrogen bonding interaction with Z-DNA from ZnADAR1 and caused an unusual phosphate backbone conformation at the G4pC5 step [10]. This unusual conformation disrupts the base-stacking interactions between the G4 and C5 bases, resulting in a downfield shift of the G42 resonance compared to the ZnADAR1–d(CG)3 complex [24]. Similarly, in the ZnADAR1–d(CG)3 complexes, the replacement of K169, K170, or R174 with an alanine may disrupt the base-stacking interactions of the G2/C3 and/or G4/C5 steps because of missing hydrogen bonds with phosphate anion. This structural feature can explain the unusual chemical shift differences of the G2z or G4z imino proton resonances in the three complexes compared to the wt-ZnADAR1–d(CG)3 complex (Supplementary Table S1).

Conclusion

The interaction of ZnADAR1 proteins containing a single mutation at residues K169, K170, N173, R174, Y177, T191, P192, P193, or W195 with a 6-bp DNA duplex [d(CG)3] was studied by 1D imino proton and 1H/15N-HSQC NMR spectroscopy. Our results were consistent with the Z-DNA binding affinities and B–Z transition activities of these mutants previously studied by various biochemical methods. This study suggests that substitution of alanine or isoleucine for residues K169, N173, or Y177 causes unusual conformational changes in the hydrophobic faces of helices α1, α2, and α3, which dramatically decrease their Z-DNA binding affinities. The 1D imino proton spectra and the chemical shift perturbation results showed that alanine substitution at residues K170, R174, T191, P192, or P193 slightly affected the Z-DNA binding affinity, but these residues did not play crucial roles in the B–Z transition. A hydrogen exchange study for the imino protons of d(CG)3 proved that the K170A- and R174A-ZnADAR1 mutants could efficiently change the B-DNA to left-handed Z-DNA via an active B–Z transition pathway, in which the G2/C5 base pair was significantly destabilized compared to wt-ZnADAR1.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.abb.2014.06.026.

References