Solution structure of the Z-DNA binding domain of PKR-like protein kinase from Carassius auratus and quantitative analyses of the intermediate complex during B–Z transition

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ABSTRACT
Z-DNA binding proteins (ZBPs) play important roles in RNA editing, innate immune response and viral infection. Structural and biophysical studies show that ZBPs initially form an intermediate complex with B-DNA for B–Z conversion. However, a comprehensive understanding of the mechanism of Z-DNA binding and B–Z transition is still lacking, due to the absence of structural information on the intermediate complex. Here, we report the solution structure of the Zα domain of the ZBP-containing protein kinase from Carassius auratus (caZPKZ). We quantitatively determined the binding affinity of caZPKZ for both B-DNA and Z-DNA and characterized its B–Z transition activity, which is modulated by varying the salt concentration. Our results suggest that the intermediate complex formed by caZαPKZ and B-DNA can be used as molecular ruler, to measure the degree to which DNA transitions to the Z isoform.

INTRODUCTION
Left-handed Z-DNA is a higher energy conformation than B-DNA and forms under conditions of high salt, negative supercoiling and complex formation with Z-DNA binding proteins (ZBPs) (1–3). ZBPs have been identified as DNA editing enzyme (ADAR1), DNA-dependent activator of interferon-regulatory factor (DAI, also known as DLM-1 and ZBP1), the viral E3L protein and a fish protein kinase containing a ZBP (PKZ) (4–7). The crystal structures of the Zα domains of human ADAR1 (hZαADAR1) (8), mouse DAI (mZαDLM1) (5) and yatapoxvirus E3L (yabZαE3L) (9), and Carassius auratus PKZ (caZαPKZ) (10) in complex with 6-base-paired (6-bp) dT(CG)3 revealed that two molecules of Zα bind to each strand of double-stranded (ds) Z-DNA, yielding 2-fold symmetry with respect to the DNA helical axis. The intermolecular interaction with Z-DNA is mediated by five residues in the β3 helix and four residues in the β-hairpin (β2-loop-β3) (Figure 1B). Among them, four residues (K34, N38, Y42 and W60; marked with asterisks in Figure 1A) show a high degree of conservation and play important roles in Zα function.

In addition, structural studies in solution suggested an active mechanism of B–Z transition of a 6-bp DNA induced by ZBPs, in which (i) the ZBP (denoted as P) binds directly to B-DNA (denoted as B); (ii) the B-DNA in the complex is converted to Z-form; and (iii) the stable ZP2 complex (the Z-form DNA denoted as Z) is produced by the addition of another P to ZP (Figure 1C) (11). In spite of these extensive structural studies (5,8–10), the detailed molecular mechanism of DNA binding and B–Z transition is still unclear due to the lack of structural data on the intermediate complexes. Therefore it is crucial to obtain structural snapshots and/or quantitative analyses of each step in the B–Z transition: B-DNA binding complex, transition complex and Z-DNA binding complex.
Figure 1. Interaction of caZαPKZ with DNA and its dependence of NaCl concentrations. (A) Multiple sequence alignment of Z-DNA binding proteins. Numbering and secondary structural elements for caZαPKZ are shown above the sequence. Yellow and gray bars indicate residues important for Z-DNA recognition and protein folding, respectively. The key aromatic residue, tyrosine, is indicated by an orange bar. The asterisks indicate four highly conserved residues which play important roles in Zα function. (B) Residues of caZαPKZ involved in intermolecular interaction with dT(CG)3 reported in a previous study (10). Intermolecular H-bonds and van der Waals contacts indicated by solid lines and dashed lines, respectively. Three water molecules in key positions within the protein–DNA interface are indicated by orange ovals. (C) Mechanism for the B→Z conformational transition of a 6-bp DNA by two ZBPs. Black arrows indicate the primary transition mechanism. (D) 1D imino proton spectra of dT(CG)3 at 35°C upon titration with caZαPKZ in NMR buffer (pH = 8.0) containing 10 (left), 100 (middle) or 250 mM NaCl (right). The resonances from B-form are labeled as G2b and G4b and those from Z-form are labeled as G2z and G4z. (E) Relative Z-DNA populations (fZ) of dT(CG)3 induced by caZαPKZ at 10 (red circle), 100 (blue square) or 250 mM NaCl (green triangle) as a function of [P]tot/[N]tot ratio. Solid lines are the best fit of the emerging G2z resonance to Equation (8).
MATERIALS AND METHODS

Sample preparation

The DNA oligomers d(CG)$_3$ and dT(CG)$_3$ were 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 for residues 1–75 of caZ$_{PKZ}$ was cloned into E. coli expression plasmid pET28a (Novagen, WI, USA). Uniformly $^{13}$C/$^{15}$N- and $^{15}$N-labeled caZ$_{PKZ}$ were obtained by growing the transformed BL21(DE3) bacteria cells in M9 medium that contained $^{15}$NH$_4$Cl and/or $^{13}$C-glucose. The $^{13}$C/$^{15}$N- and $^{15}$N-labeled caZ$_{PKZ}$ proteins were purified with a Ni–NTA affinity column and a Sephacryl S-100 gel filtration column (GE Healthcare, USA) on a GE AKTA Prime Plus. The amount of DNA was represented as the concentration of the double-stranded sample. The DNA and protein samples were dissolved in a 90% H$_2$O/10% D$_2$O NMR buffer containing 10 mM sodium phosphate (pH = 6.0 or 8.0) and NaCl with concentration of 10, 100 or 250 mM.

NMR experiments

All of the $^1$H, $^{13}$C and $^{15}$N NMR experiments were performed on an Agilent DD2 700-MHz spectrometer (GNN, Jinju) or a Bruker Avance-III 800-MHz spectrometer (KBSI, Ochang) equipped with a triple-resonance cryogenic probe. All three-dimensional (3D) triple resonance experiments were carried out with 1.0 mM $^{13}$C/$^{15}$N-labeled caZ$_{PKZ}$ protein. The imino proton and $^1$H $^{15}$N-HSQC spectra were obtained for complexes prepared by addition of $^{15}$N-labeled caZ$_{PKZ}$ to 0.1–0.2 mM DNA or addition of DNA to 1 mM $^{15}$N-labeled caZ$_{PKZ}$. One dimensional (1D) NMR data were processed with either VNMR J (Agilent, Santa Clara, CA, USA) or FELIX2004 (FELIX NMR, San Diego, CA, USA) software, while the two-dimensional and 3D data were processed with NMRPIPE (19) and analyzed with Sparky (20). External 2–2-dimethyl-2-silapentane-5-sulfonate was used for the $^1$H, $^{13}$C and $^{15}$N references.

$^1$H, $^{13}$C and $^{15}$N resonance assignments for caZ$_{PKZ}$ were obtained from the following 3D experiments in 10% D$_2$O/90% H$_2$O containing 10 mM sodium phosphate (pH 8.0) and 100 mM NaCl: CBC(CO)NH, HNCA, HNCA(CB)CO(NH), CB(CCGCDCE)HE, HHCCH-TOCSY, NOESY-$^1$H/$^{15}$N-HSQC, NOESY-$^1$H/$^{15}$N-HSQC and TOCSY-$^1$H/$^{15}$N-HSQC. The average chemical shift differences of the amide proton and nitrogen resonances between the two states were calculated by Equation (1):

$$\Delta \delta_{\text{avg}} = \sqrt{(\Delta \delta_H)^2 + (\Delta \delta_N/5.88)^2}$$

(1)

where $\Delta \delta_H$ and $\Delta \delta_N$ are the chemical shift differences of the amide proton and nitrogen resonances, respectively.

Solution structure calculation

The inter-proton distance restraints were extracted from NOESY-$^1$H/$^{15}$N-HSQC and NOESY-$^1$H/$^{13}$C-HSQC spectra. Backbone dihedral angle restraints were generated using TALOS+ (21). Only phi ($\phi$) and psi ($\psi$) angle restraints
which qualified as ‘good’ predictions from TALOS+ were used in the structure calculation. Hydrogen-bonds were introduced as a pair of distance restraints based on nuclear Overhauser effect (NOE) analysis in combination with the prediction of protein secondary structural elements using the software CSI (22). Structure calculations were initially performed with CYANA 2.1, which combines automated assignment of NOE cross-peaks and structure calculation. On the basis of distance restraints derived from CYANA output, further structure calculations were carried out using CNS 1.3 in explicit solvent using the RECOORD protocol (23–25). The 10 lowest-energy structures were validated by PROCHECK-NMR (26). Structures were visualized using the program Discovery Studio 4.1 (BIOVIA, San Diego, CA, USA). Structural statistics are presented in Table 1.

Nitrogen Carr-Purcell-Meiboom-Gill (CPMG) relaxation dispersion

The 15N amide Carr-Purcell-Meiboom-Gill (CPMG) relaxation dispersion experiments were performed using free 15N-labeled caZαPKZ and 15N-labeled caZαPKZ complexed with dT(CG)3 at 35°C. Experiments employed a constant relaxation delay \( T_{\text{relax}} \) of 60 ms and 11 values of \( ν_{\text{CPMG}} \), ranging from 33 to 900 Hz, \( τ_\text{CP} \) is the decay between consecutive pulses. Transverse relaxation rates \( R_{\text{2,eff}} \) were calculated for each cross-peak signal at each value of CPMG.

\[
R_{\text{2,eff}}(\nu_{\text{CPMG}}) = -\frac{1}{T_{\text{relax}}} \ln \left( \frac{I(\nu_{\text{CPMG}})}{I_0} \right) 
\]

where \( I(\nu_{\text{CPMG}}) \) and \( I_0 \) are the peak intensities at values of \( \nu_{\text{CPMG}} \) with 60 and 0 ms, respectively. For evaluation of average and standard deviation of \( R_{\text{2,eff}} \) values, three or four different datasets were measured.

In the free caZαPKZ, the relaxation dispersions derived from residues in fast exchange on the NMR chemical shift timescale were fitted to (27):

\[
R_{\text{eff}}^{\text{free}}(\nu_{\text{CPMG}}) = R_0^\phi + \frac{\Phi_{\text{ex,f}}}{k_{\text{ex,f}}} \left[ 1 - \frac{4\nu_{\text{CPMG}}}{k_{\text{ex,f}}} \tanh \left( \frac{k_{\text{ex,f}}}{4\nu_{\text{CPMG}}} \right) \right]
\]

where \( R_0^\phi \) is the intrinsic transverse relaxation rate; \( \Phi_{\text{ex,f}} = \Phi_{\text{ex,b}} \left( \Delta δ_{\text{obs,b}} \right)^2 \), where \( \Delta δ_{\text{obs,b}} \) is chemical shift difference between states A and B and \( \Phi_{\text{ex,f}} \) and \( \Phi_{\text{ex,b}} \) are the relative populations of states A and B, respectively; and \( k_{\text{ex,f}} \) and \( k_{\text{ex,b}} \) are the exchange rate between states A and B. The caZαPKZ in the complex with Z-DNA shows two kinds of independent exchange processes, the conformational exchange of free protein and the association/dissociation of Z-DNA. In this case, the relaxation dispersion data of the caZαPKZ complexed with dT(CG)3 \( (R_{\text{2,eff,comp}}) \) could be expressed by Equation (4):

\[
R_{\text{2,eff,comp}}^{\text{comp}}(\nu_{\text{CPMG}}) = R_0^\phi + \frac{\Phi_{\text{ex,b}}}{k_{\text{ex,b}}} \left[ 1 - \frac{4\nu_{\text{CPMG}}}{k_{\text{ex,b}}} \tanh \left( \frac{k_{\text{ex,b}}}{4\nu_{\text{CPMG}}} \right) \right]
\]

where \( R_0^\phi \) is the intrinsic transverse relaxation rate; \( \Phi_{\text{ex,b}} = \Phi_{\text{ex,f}} \left( \Delta δ_{\text{obs,b}} \right)^2 \), where \( \Delta δ_{\text{obs,b}} \) is chemical shift difference between the bound and unbound states and \( \Phi_{\text{ex,b}} \) and \( \Phi_{\text{ex,f}} \) are the relative populations of the bound and unbound states, respectively; and \( k_{\text{ex,b}} \), the exchange rate between the bound and unbound states (28).

Binding models

Accordingly, the HSQC titration curves were analyzed by assuming an active model of B-Z transition (Figure 1C), where P is the free forms of caZαPKZ, BP and ZP are the singly bound forms to B-DNA and Z-DNA, respectively, ZP2 is the doubly bound form to Z-DNA, and B is the B-form of free dT(CG)3. The concentrations of B, BP, ZP and ZP2 forms, [B], [BP], [ZP] and [ZP2], respectively, are described as:

\[
\begin{align*}
[B] &= [N]_{\text{tot}} + K_{\text{d,BP}} \frac{K_{\text{d,ZP}}}{K_{\text{d,BP}} + K_{\text{d,ZP}}} [P] + K_{\text{ZP1}} [P]^2 \\
[BP] &= [N]_{\text{tot}} + K_{\text{d,BP}} \frac{K_{\text{d,ZP}}} {K_{\text{d,BP}} + K_{\text{d,ZP}}} [P] + K_{\text{ZP1}} [P]^2 \\
[ZP] &= [N]_{\text{tot}} \frac{K_{\text{d,BP}} K_{\text{d,ZP}}} {K_{\text{d,BP}} + K_{\text{d,ZP}}} [P] + K_{\text{ZP1}} [P]^2 \\
[ZP2] &= [N]_{\text{tot}} + K_{\text{d,BP}} K_{\text{d,ZP}} [P] + K_{\text{ZP2}} [P]^2 
\end{align*}
\]

where \([N]_{\text{tot}}\) is the total concentration of DNA duplex; \(K_{\text{d,BP}}\) and \(K_{\text{d,ZP2}}\) are the dissociation constants for the BP and ZP2 complexes, respectively; \(K_{\text{ZP1}}\) (= \([ZP]/[BP]\)) is the equilibrium constant between BP and ZP forms; and \([P]\) is the concentration of the free caZαPKZ, which is a solution of the following cubic equation:

\[
[P]^3 + a[P]^2 + b[P] + c = 0
\]

where

\[
a = 2[\text{[N]}_{\text{tot}} - [\text{[P]}_{\text{tot}}] + \left( 1 + \frac{1}{K_{\text{ZP1}}} \right) K_{\text{d,ZP2}}
\]

\[
b = \left( 1 + \frac{1}{K_{\text{ZP1}}} \right) K_{\text{d,ZP2}}\left( [\text{[N]}_{\text{tot}} - [\text{[P]}_{\text{tot}}] + \frac{K_{\text{d,BP}} K_{\text{d,ZP}}}{K_{\text{ZP1}}} \right)
\]

\[
c = -\frac{K_{\text{d,BP}} K_{\text{d,ZP}}}{K_{\text{ZP1}}} [\text{[P]}_{\text{tot}}]
\]

where \([\text{[P]}_{\text{tot}}] \) is the total concentration of caZαPKZ. The closed-form solution of Equation (6) has been reported (29):

\[
[P] = -\frac{\alpha}{3} + \frac{2}{3} \sqrt{\alpha^2 - 3b \cos \frac{\theta}{3}}
\]

where,

\[
\theta = \arccos \frac{-2a^2 + 9ab - 27c}{2\sqrt{(a^2 - 3b)^3}}
\]

The observed \(^1\text{H}\) and \(^15\text{N}\) chemical shift difference referred to the free caZαPKZ, \(\Delta \delta_{\text{obs}}\), is described as:

\[
\Delta \delta_{\text{obs}} = \frac{[\text{[BP]}]}{[\text{[P]}_{\text{tot}}]} \Delta \delta_B + \frac{[\text{[ZP]}]}{[\text{[P]}_{\text{tot}}]} \Delta \delta_Z
\]
where $\Delta \delta_H$ and $\Delta \delta_Z$ are the $^1$H and $^{15}$N chemical shift differences of the B-DNA- and Z-DNA-bound forms relative to the free form, respectively. The relative Z-DNA populations ($f_Z$) could be determined from the integration of new resonances in the $^{31}$P NMR or imino proton spectra, which provide the same results (11). The observed $f_Z$ value determined from imino proton resonances is described as:

$$f_Z = \frac{[\text{ZP}] + [\text{ZP}_2]}{[\text{N}]_{\text{tot}}}$$ (9)

Hydrogen exchange rate measurement

The apparent longitudinal relaxation rate constants ($R_{1a} = 1/T_{1a}$) of the imino protons of free and bound DNA were determined by semi-selective inversion recovery 1D NMR experiments. The hydrogen exchange rate constants ($k_{ex}$) of the imino protons were measured by a water magnetization transfer experiment with 20 different delay times (30,31). The $k_{ex}$ values for the imino protons were determined by fitting the data to Equation (10):

$$\frac{I_0 - I(t)}{I_0} = 2 \frac{k_{ex}}{R_{1w} - R_{1a}} \left( e^{-R_{1a}t} - e^{-R_{1w}t} \right)$$ (10)

where $I_0$ and $I(t)$ are the peak intensities of the imino proton at times zero and $t$, respectively, and $R_{1a}$ and $R_{1w}$ are the apparent longitudinal relaxation rate constants for the imino proton and water, respectively (30–32).

RESULTS

Titration of caZPKZ into dT(CG)$_3$ under various [NaCl]

Figure 1D shows the changes in the imino-proton spectra of dT(CG)$_3$ upon titration with caZPKZ at 35°C. The relative populations of Z-DNA ($f_Z$) in dT(CG)$_3$ were determined by integration of the new G2z resonance, as a function of the total protein: total DNA duplex ($[P]_{\text{tot}}/[N]_{\text{tot}}$) ratio (Figure 1E). CaZPKZ exhibited a severe decrement in B–Z transition activity as [NaCl] increased. Most of the dT(CG)$_3$ was in the Z-conformation at $[P]_{\text{tot}}/[N]_{\text{tot}} > 2.0$ at 10 mM NaCl. However, at [NaCl] = 100 mM, only about 67% of dT(CG)$_3$ displayed the Z-DNA conformation at $[P]_{\text{tot}}/[N]_{\text{tot}} = 2.0$ (Figure 1E), in contrast to previous findings that most of d(CG)$_3$ was converted to Z-DNA by hZADAR1 (11) and yabZPKZ (13) under the same conditions. Interestingly, when [NaCl] increased up to 250 mM, caZPKZ showed extremely low B–Z transition activity to dT(CG)$_3$ (Figure 1E).

Solution structure of free caZPKZ

In order to elucidate detailed structural information for free caZPKZ, the solution structure of free caZPKZ was determined by restrained molecular dynamics calculations using 878 distance restraints and 109 dihedral angle restraints collected at 100 mM NaCl (Table 1). A final set of 10 lowest energy structures was selected from 100 calculations, with NOE violations larger than 0.5 Å and 5° for the NOEs and dihedral angles, respectively (Figure 2A). The RMSD values for the backbone atoms in the structured region was calculated to be 0.57 ± 0.18 Å. Free caZPKZ is composed of three $\alpha$-helices ($\alpha_1$, $\alpha_2$ and $\alpha_3$) and three $\beta$-strands ($\beta_1$, $\beta_2$ and $\beta_3$) (Figure 2A). The ensemble of the 10 lowest energy structures shows that only the L4 loop in the $\beta$-hairpin (residues 53–57) is not well converged whereas all other loops are tightly structured (Figure 2A). Two consecutive prolines (P57 and P58) which have only limited numbers of restraints hinder to determine the precise orientation of the $\beta$-hairpin in solution structures. Figure 2B shows the superimposition of the lowest energy structure of free caZPKZ and the crystal structure of caZPKZ–dT(CG)$_3$ complex (10), where the $\beta$-hairpin exhibits bigger differences while no significant structural deviations were observed in other structural regions. The crystal structure showed that K56 sidechain in the $\beta$-hairpin of caZPKZ is involved in H-bonding with the phosphate of Z-DNA in the caZPKZ–dT(CG)$_3$ complex (10) (Figure 2B). It might be possible that the orientation of the $\beta$-hairpin is restrained when the sidechain of K56 interacts with the backbone of Z-DNA.
Chemical shift changes in caZα PKZ upon binding to dT(CG)$_3$

In the $^1$H/$^{15}$N-HSQC spectra of free caZαPKZ and caZαPKZ–dT(CG)$_3$ at 35°C, the amide resonances for several residues (N38, R39 and Y42) of the α3 helix disappeared altogether (Supplementary Figure S1), indicating the direct interaction of the sidechains of caZαPKZ with the phosphate backbone of dT(CG)$_3$ as reported in the previous crystal structural study (10). As expected from the imino proton spectra at 250 mM NaCl, few chemical shift changes occurred upon exposure to dT(CG)$_3$ (Supplementary Figure S1).

To further clarify the chemical shift perturbation results, the $^1$H/$^{15}$N-HSQC spectra of caZαPKZ were acquired at 35°C as a function of the [N$_{tot}$/P$_{tot}$] ratio, where most amide cross-peaks showed significant movement (Supplementary Figure S2). Interestingly, the cross-peaks of some residues changed the direction of their movement after achieving a certain position (Figure 3A and Supplementary Figure S2), indicating the presence of at least two binding modes. We analyzed the HSQC titration curves as well as the relative Z-DNA populations ($f_z$) assuming an active B–Z transition model (Figure 1C). $K_{d, BP}$ and $K_{d, ZP2}$ are the dissociation constants of the BP and ZP$_2$ complexes, respectively, and $K_{BZ.1}$ (=[ZP]/[BP]) is the equilibrium constant between the ZP and BP complexes. Because fitting each titration curve independently did not give reliable $K_d$ values, all titration curves were fitted globally. During global fitting, we have fitted simultaneously all $^1$H and $^{15}$N titration curves with Equation (8) and the $f_z$ data with Equation (9) to obtain accurate $K_d$ values (Figure 3B–D). At 10 mM NaCl, the global fitting gave $K_{d,BP}$ and $K_{d,ZP2}$ of 28 ± 17 and 345 ± 79 nM, respectively, and $K_{BZ.1}$ of 0.87 ± 0.01 (Table 2 and Figure 3B and C). The dataset at 100 mM NaCl was globally fitted to obtain $K_{d,BP}$ and $K_{d,ZP2}$ values of 16.4 ± 0.8 and 8.76 ± 0.67 μM, respectively, and $K_{BZ.1}$ of 0.19 ± 0.01 (Table 2 and Figure 3B and D). These results indicate that the incrementation of [NaCl] from 10 to 100 mM leads to 600- and 25-fold larger $K_d$ of caZαPKZ for B-DNA and Z-DNA binding, respectively, and 4.6-fold lower B–Z transition activity in the complex form (Table 2). The titration data at 250 mM NaCl could not be analyzed based on an active B–Z transition model, because of the extremely smaller $K_{BZ.1}$.

Chemical shift differences in caZα PKZ bound to B-DNA and Z-DNA

In addition to the dissociation constants, the global fitting method also provides the $^1$H and $^{15}$N chemical shift differences between the free and the bound forms for both B-DNA and Z-DNA binding (Supplementary Figure S3). The combined averages of $^1$H and $^{15}$N chemical shift changes ($\Delta \delta_{avg}$) were determined for each residue to represent effects of binding to B-DNA and Z-DNA (Figure 4A). The chemical shift information for residues N38, R39, L41, Y42 and L44 in the α3 helix could not be collected because these resonances disappeared or became very weak during titration upon DNA. At 10 mM NaCl, both B-DNA and Z-DNA binding of caZαPKZ caused similar chemical shift perturbations, such that significant chemical shift changes were observed in the α3 helix as well as in the β1–α2 and β-hairpin regions (Figure 4A). However, at 100 mM NaCl, the B-DNA and Z-DNA binding of caZαPKZ exhibited completely different chemical shift perturbation results from each other (Figure 4A). For the Z-DNA binding, a large chemical shift changes were observed for the α3, β1–α2 and β-hairpin regions, similar to data at 10 mM NaCl (Figure 4A and B). On the other hand, the B-DNA binding affected I30 in L2; S35, Q45 and R46 in α3; and N54 and V59 in the β-hairpin with $\Delta \delta_{avg} > 0.06$ ppm (Figure 4A and C). These results meant that the B-DNA binding state of caZαPKZ exhibited distinct structural features under high and low salt conditions, which might be related to reduced B–Z transition activity at higher [NaCl].

The superimposed $^3$H/$^{15}$N-HSQC spectra of free caZαPKZ at 10, 100 or 250 mM NaCl at 25°C are shown in Supplementary Figure S4. Significant chemical shift differences were observed for residues in the α2 and α3 regions with increasing [NaCl] from 10 to 100 mM (Figure 4D). However, all residues in the Z-DNA binding complex showed little chemical shift changes ($\Delta \delta_{avg} < 0.05$ ppm).
upon change of [NaCl] (Figure 4D). For example, the A23 and L24 amide signals in the Z-DNA binding complex were located at the almost same position in the spectra (red and orange peaks in Figure 4E), whereas they showed the significant chemical shift differences in the free form (green and brown peaks in Figure 4E). These results indicate that the Z-DNA binding complex of caZPKZ maintains almost the same structural features regardless of salt concentration, contrary to free caZPKZ.

15N Carr-Purcell-Meiboom-Gill (CPMG) relaxation dispersion experiment on caZPKZ bound to dT(CG)3

The rate constants for association and dissociation of caZPKZ with DNA were determined using NMR 15N backbone amide CPMG relaxation dispersion experiments (33). According to the concentrations of each state calculated using Equation (4) as a function of [N]tot/[P]tot, free P and complex ZP2 are the only two major conformational states when [N]tot/[P]tot << 0.5 (Supplementary Figure S5). Thus we analyzed the CPMG data using a two-state model of conformational exchange, where the ZP and ZP2 are considered as the ligand (L) and complex (PL), respectively (Figure 5A). In this model, the conformation exchange rate (kex) is given by kex = kon,ZP[ZP] + koff,ZP2, where k_{on,ZP} is the association rate of ZP, k_{off,ZP2} is the dissociation rate of ZP2 and [ZP] is the concentration of ZP (Figure 5A). Interestingly, several residues of free caZPKZ exhibited conformational exchange with kex of 550 ± 296 and 684 ± 153 s⁻¹ at 10 and 100 mM NaCl, respectively (Figure 5B). Thus, in order to minimize the effect of conformational exchange of free caZPKZ, the transverse relaxation rate differences between the caZPKZ−dT(CG)3 complex and free caZPKZ (R_{2,eff}−comp−R_{2,eff}−free) were fitted globally using Equation (4). The Δω values were determined from global fitting of the 1H/15N titration data and the relative population of free caZPKZ, Pfree (= [P]/[P]tot) was calculated by p_{free} = 1 − Δω_{obs}/Δω (Figure 5C). Even though we used these fixed Δω and p_{free} values for fitting of the CPMG data, the all CPMG data were globally well fitted (Figure 5D). It indicates that the conformation exchange for the association/dissociation of Z-DNA exhibits the single k_{ex} value through all residues. At 10 mM NaCl, the CPMG

Table 2. Dissociation constants (K_{d,BP} and K_{d,ZP2}) for B-DNA and Z-DNA binding, equilibrium constant for B–Z transition (K_{BZ,1}) and association/dissociation rate constants (k_{on,ZP} and k_{off,ZP2}) for Z-DNA binding of caZPKZ with a 6-bp DNA

| DNA | pH | [NaCl] (mM) | K_{d,BP} (μM) | K_{d,ZP2} (μM) | K_{BZ,1} | k_{on,ZP} (×10⁸ M⁻¹s⁻¹) | k_{off,ZP2} (s⁻¹) |
|-----|----|-------------|--------------|---------------|----------|-----------------|-----------------|
| dT(GC)₃ | 6.0 | 10 | 0.028 ± 0.017 | 0.345 ± 0.079 | 0.87 ± 0.03 | 19.6 ± 1.2b | 675 ± 43b |
|       |     | 100 | 16.4 ± 0.8 | 8.76 ± 0.67 | 0.19 ± 0.02 | 1.56 ± 0.03b | 1381 ± 30b |
|       | 8.0 | 10 | 0.157 ± 0.021 | 0.129 ± 0.074 | 1.18 ± 0.03 | 0.87 ± 0.03 |
|       |     | 100 | 5.41 ± 0.66 | 2.41 ± 0.37 | 0.18 ± 0.02 | 0.87 ± 0.03 |
| d(GC)₃ | 8.0 | 10 | 5.18 ± 2.43 | 1.79 ± 0.95 | 0.11 ± 0.05 | 0.87 ± 0.03 |

aFrom CPMG data with P_{free} = 0.873.
bFrom CPMG data with P_{free} = 0.891.
Figure 4. DNA binding patterns differ with salt concentration and DNA conformation. (A) Histograms of the Δδ_avg values of 15N-caZPKZ for the B-DNA and Z-DNA binding at 10 (left) or 100 mM NaCl (right). Residues whose cross-peaks disappear or become very weak during titration are represented with green square symbols. The asterisks indicate residues whose cross-peaks overlap with other resonances during titration. (B and C) Mapping the location of the residues having large Δδ_avg onto the NMR structure of free caZPKZ for the (B) Z-DNA and (C) B-DNA binding at 10 (left) or 100 mM NaCl (right). The colors used to illustrate the Δδ_avg are: red or blue, >0.18 ppm; orange or cyan, 0.12–0.18 ppm; and yellow or pale green, 0.08–0.12 ppm (the same color coding is used in panel A). In both panels, the purple spheres indicate residues whose cross-peaks disappear or become very weak during titration. (D) The average chemical shift differences (Δδ_avg) between [NaCl] of 10 and 100 mM for free caZPKZ (upper) and caZPKZ complexed with B-DNA (middle) and Z-DNA (lower). (E) The calculated 1H/15N-HSQC cross-peaks of caZPKZ complexed with B-DNA and Z-DNA in buffer containing 10 or 100 mM NaCl.
dataset with $p_{\text{free}} = 0.873$ was globally fit to obtain the $k_{\text{ex}}$ of $774 \pm 49$ s$^{-1}$, which was used to calculate the $k_{\text{on, ZP}}$ and $k_{\text{off, ZP}}$ of $98 \pm 6$ and $675 \pm 43$ s$^{-1}$, respectively (using $k_{\text{off, ZP}} = k_{\text{ex}} \times p_{\text{free}}$) and the $k_{\text{on, ZP}}$ of $(1.96 \pm 0.12) \times 10^{9}$ M$^{-1}$s$^{-1}$ (using $k_{\text{on, ZP}} = k_{\text{off, ZP}}/K_{d, ZP}$) (Table 2 and Figure 5D). In the case that [NaCl] = 100 mM, the dataset at $p_{\text{free}} = 0.891$ was globally fitted to obtain the $k_{\text{ex}}$ of $1550 \pm 34$ s$^{-1}$ and the $k_{\text{off, ZP}}$ and $k_{\text{on, ZP}}$ of $1381 \pm 30$ and $(1.56 \pm 0.03) \times 10^{8}$ M$^{-1}$s$^{-1}$, respectively (Table 2 and Figure 5D). These results indicate that, as [NaCl] increased from 10 to 100 mM, the 13-fold slower $k_{\text{on, ZP}}$ and 2-fold faster $k_{\text{off, ZP}}$ resulted in a 25-fold larger $K_{d, ZP}$.

**H-bonding interaction of K56 with Z-DNA phosphate**

The crystal structure of the caZPKZ–dT(CG)$_3$ complex showed that the sidechain of K56 exhibited an unusual H-bonding interaction with the TopCl phosphate of Z-DNA (Figure 1B) (10). Thus, in order to understand the role of this intermolecular H-bonding interaction, NMR titrations of caZPKZ into d(CG)$_3$ were performed (Figure 6A). At 10 mM NaCl, only 57% of d(CG)$_3$ was converted to Z-DNA by caZPKZ at $[P]_{\text{tot}}/[N]_{\text{tot}} = 2.0$ (Figure 6B), whereas most of d(T(CG)$_3$) exhibited the Z-DNA conformation at $[P]_{\text{tot}}/[N]_{\text{tot}} \geq 2.0$ (Figure 1E). A similar decrement in B–Z transition activity was also observed at 100 mM NaCl (Figure 6B). We fitted simultaneously the $^1$H and $^{15}$N titration curves of caZPKZ and the $f_z$ data of d(CG)$_3$ and d(T(CG)$_3$) at pH 8.0 to compare the B–Z transition activities of these two DNA by caZPKZ (Supplementary Figure S6). The global fitting showed that d(CG)$_3$ has 33- and 14-fold larger values of $K_{d, BP}$ and $K_{d, ZP}$, respectively, and a 10-fold smaller $K_{BZ,1}$ than d(T(CG)$_3$) at 10 mM NaCl (Table 2). When [NaCl] = 100 mM, the $^1$H/$^{15}$N-HSQC titration data of d(CG)$_3$ were not fitted well because of its very low B–Z transition activity (meaning $K_{BZ,1} << 1$). These results suggest that the intermolecular H-bonding interaction of K56 with the phosphate backbone of Z-DNA significantly contributes not only to DNA binding of caZPKZ but also to its B–Z transition activity.

The hydrogen exchange rate constants ($k_{\text{ex}}$) for the imino protons of both free and caZPKZ-bound d(T(CG)$_3$) and d(CG)$_3$ were determined at 35°C. The G2h and G4b protons of free d(T(CG)$_3$) have significantly smaller $k_{\text{ex}}$ values compared to those of free d(CG)$_3$ (Figure 6C and D), in-
indicating that the flanking T residue of dT(CG)3 leads to greater stabilization of the central G-C base-pairs. In the caZαPKZ−dT(CG)3 complex, the $k_{ex}$ of G4z are significantly smaller than those of free dT(CG)3, whereas the G2z imino protons have slightly larger $k_{ex}$ than the corresponding G2b protons (Figure 6C). Surprisingly, the G2z in the caZαPKZ−d(CG)3 complex has significantly larger $k_{ex}$ than in the caZαPKZ−dT(CG)3 complex (Figure 6D), consistent with severe line-broadening of the G2z imino resonance (Figure 6A). These results indicate that the intermolecular H-bonding interaction of K56 plays an important role in stabilization of the G2-C5 base-pair in Z-DNA.

**DISCUSSION**

The caZαPKZ protein requires low salt concentration for full B-Z transition activity, although its overall structure and interactions with Z-DNA are similar to other ZBPs. It has been reported that the typical intracellular salt concentration of fresh water fish is maintained at as few as 10 mM (34,35), and goldfish showed low salinity tolerance (<20 ppt) (36). Thus the salt-dependent B–Z transition activity of caZαPKZ is thought to reflect the natural environmental conditions of goldfish. We have undertaken a structural analysis of protein–DNA interactions during B–Z transition of a 6-bp DNA by caZαPKZ, in order to understand the salt-depency of ZBP activity. The NMR chemical shift perturbation (Supplementary Figure S1) and crystal structure analyses (10) can only illustrate the interactions of ZBP with Z-DNA in the final caZαPKZ−Z-DNA (ZP2) complex. However, by applying global analysis on the titration curves, we are able to provide structural information on caZαPKZ not only in the Z-DNA binding complex but also in the B-DNA binding complex that is the first intermediate structure in the B-Z transition pathway (Supplementary Figure S3). Global analysis gave the $K_d$ of caZαPKZ for the BP (28 nM) and ZP2 complexes (345 nM) under 10 mM NaCl condition (Table 2), which have orders of magnitude similar to the previous $K_d$ (851 nM) determined by bio-layer interferometry (10). The values of $k_{on, ZP}$ ($1.96 × 10^9$ M$^{-1}$s$^{-1}$) and $k_{off, ZP2}$ ($675$ s$^{-1}$), which are supported by fast exchange behavior in the $^1$H/$^15$N-HSQC spectra during titration (Supplementary Figure S2), are consistent with the association process of $10^9$–$10^{10}$ M$^{-1}$s$^{-1}$ reported for protein–nucleic acid interactions (15,37–39). Taken together, we conclude that the analysis of CPMG data combined with global fitting of titration curves is the most effective method to estimate accurate dissociation constants as well as dissociation/association rate constants in a multi-site protein–DNA binding system including conformational changes of DNA and/or proteins.

The increase of [NaCl] from 10 to 100 mM exhibits a larger effect on the $k_{on,ZP}$ (13-fold larger) than $k_{off,ZP2}$ (2-
However, as [NaCl] increased to 100 mM, the causing force to culminate in a 25-fold larger activation energy difference ($\Delta G^\ddagger_{BP}$), where $K$ is the dissociation constant for DNA binding ($K_{d, BP}$ or $K_{d, ZP}$) or $\Delta G^\ddagger = -RT\ln K_{BP, ZP}$, where $K_{BP, ZP}$ is the equilibrium constant for B-Z transition. The activation energy difference ($\Delta \Delta G^\ddagger_{ZP}$) for the Z-DNA binding of caZapKZ between 10 and 100 mM NaCl condition was calculated using the equation, $\Delta \Delta G^\ddagger_{ZP} = \Delta G^\ddagger_{ZP, 100mM} - \Delta G^\ddagger_{ZP, 10mM} = -RT\ln(k_{on,ZP, 100mM}/k_{on,ZP, 10mM})$, where $k_{on,ZP, 100mM}$ and $k_{on,ZP, 10mM}$ are the association rate constants for Z-DNA binding of caZapKZ at 10 and 100 mM NaCl, respectively.

![Figure 7](image_url)

**Figure 7.** Quantitative description of the energy landscape of the first (left) and second DNA binding (right) of caZapKZ at 10 (red) or 100 mM NaCl (blue). Gibbs free energies for the DNA binding and B-Z transition steps were calculated using the equation, $\Delta G = RT\ln K_d$, where $R$ is the gas constant and $K_d$ is the dissociation constant for DNA binding ($K_{d, BP}$ or $K_{d, ZP}$) or $\Delta G^\ddagger = -RT\ln K_{BP, ZP}$, where $K_{BP, ZP}$ is the equilibrium constant for B-Z transition. The activation energy difference ($\Delta \Delta G^\ddagger_{ZP}$) for the Z-DNA binding of caZapKZ between 10 and 100 mM NaCl condition was calculated using the equation, $\Delta \Delta G^\ddagger_{ZP} = \Delta G^\ddagger_{ZP, 100mM} - \Delta G^\ddagger_{ZP, 10mM} = -RT\ln(k_{on,ZP, 100mM}/k_{on,ZP, 10mM})$, where $k_{on,ZP, 100mM}$ and $k_{on,ZP, 10mM}$ are the association rate constants for Z-DNA binding of caZapKZ at 10 and 100 mM NaCl, respectively.

d fold larger), and this resulted in a 25-fold larger $K_{d, ZP}$ (Table 2). Similar results were observed for the binding of Fyn SH3 domain to substrate peptides (40). The Gibbs free energies for the formation of ZP$_2$ can be calculated using the equation $\Delta G^\ddagger_{ZP} = RT\ln K_{d, ZP}$. The results revealed that the difference between the $\Delta G^\ddagger_{ZP}$ values at 10 and 100 mM NaCl ($\Delta \Delta G^\ddagger_{ZP} = \Delta G^\ddagger_{ZP, 100mM} - \Delta G^\ddagger_{ZP, 10mM}$) is $\pm 2.0$ kcal/mol (Figure 7). The activation energy differences for association ($\Delta G^\ddagger_{ZP, on}$) and dissociation ($\Delta G^\ddagger_{ZP, off}$) of the ZP$_2$ complex can be evaluated using the equations, $\Delta G^\ddagger_{ZP, on} = -RT\ln(k_{on,ZP, 100mM}/k_{off,ZP, 100mM})$ and $\Delta G^\ddagger_{ZP, off} = -RT\ln(k_{off,ZP, 100mM}/k_{on,ZP, 100mM})$, respectively. The CPMG data revealed that the $\Delta G^\ddagger_{ZP}$ value was $1.6$ kcal/mol whereas $\Delta \Delta G^\ddagger_{ZP}$ was only $-0.4$ kcal/mol (Figure 7), which could be explained by the structural feature that, in the Z-DNA binding complex, caZapKZ maintains almost the same backbone conformations regardless of salt concentration (Figure 4D). However, the free form of caZapKZ shows unusual conformational exchange (Figure 5B), which displays distinct $^1$H/$^15$N-HSQC spectra with varying salt concentration (Supplementary Figure S4). This structural feature is consistent with the CPMG data showing that, in spite of a 2-fold larger $k_{off,ZP, 2}$, the $K_{d, ZP}$ value increased 25× larger as [NaCl] increased from 10 to 100 mM (Table 2). Thus, our study suggested that increasing the ionic strength more strongly interferes with the association of ZP with caZapKZ via the intermolecular electrostatic interactions (related to $k_{on,ZP}$) rather than the dissociation of ZP$_2$ (related to $k_{off,ZP, 2}$).

Surprisingly, we found that the structural features of the B-DNA binding complexes (BP) of caZapKZ at 10 and 100 mM NaCl are completely different from each other (Figure 4D). At 10 mM NaCl, the α3, β1–α2 and β-hairpin regions of caZapKZ participate in strong intermolecular interactions with B-DNA (Figure 4B). This intermolecular interaction in the BP complex is able to provide efficient driving force to cause the B–Z conformational change of DNA. However, as [NaCl] increased to 100 mM, the caZapKZ binds to B-DNA mainly through the α3 helix, with a partial contribution of the β-hairpin (Figure 4B). Global analysis found that increasing [NaCl] from 10 to 100 mM more significantly reduced the Gibbs free energies for the formation of BP with $\Delta G^\ddagger_{BP}$ of 3.9 kcal/mol, which is 2-fold larger than the $\Delta \Delta G^\ddagger_{ZP}$ value (Figure 7). It is reported that the activity of nitrogen regulatory protein C can be modulated by mutations or BeF$_3^−$ , which drive the equilibrium toward the active state through destabilizing the inactive state and stabilizing the active state, respectively (41). Assuming that caZapKZ binds to B-DNA to form a fully active BP conformation at low salt concentration, increasing the ionic strength screens the intermolecular electrostatic interactions, which participates in formation of the active BP conformation, and then the conformational equilibrium is driven toward the inactive state. As shown in Figure 4E, the ~25–40% chemical shift movements of caZapKZ upon B-DNA binding, reflecting a lower population of the active BP conformation, can explain the relatively lower B–Z transition activity (4.5-fold smaller $K_{BZ, 1}$) (Table 1). Thus, the HSQC spectra of the intermediate BP complex derived from our global analysis could be used as a molecular ruler to determine the degree of B–Z transition activity of caZapKZ (Figure 4E).

The hydrogen exchange data of the imino protons indicates that the G-C base-pairs in the caZapKZ–dT(CG)$_3$ complex are less stable compared to the hZαADAR1–d(CG)$_3$ complex (Figure 6C and D). This might be caused by the lack of H-bonding interaction of caZapKZ with the C5pG6 phosphate of DNA (Figure 1B), which is provided by the K170 sidechain of hZαADAR1 (8). Under low salt conditions, this H-bonding interaction of ZBP rarely affects the DNA binding as well as B–Z transition. In contrast, the H-bonding of K56 with DNA phosphate contributes to not only stabilization of base-pairs in Z-DNA but also DNA binding and B–Z transition (Figure 6). Because the backbone of the β-hairpin in free caZapKZ is distinct from that of the complex structure (Figure 2B), this interaction plays
an important role in stabilizing the contact of the β-hairpin with Z-DNA in order to achieve full activity of the ZBP. In hZARADAR1, both the free and complex forms have similar β-hairpin structures (8,42) and thus this interaction is not crucial for the function of the human protein.

In summary, we have performed a structural analysis of the interaction of ZBP caZPKZ with DNA during B–Z transition. The solution structure of free caZPKZ revealed that the overall structure is very similar to the caZPKZ–dT(CG)3 complex while the β-hairpin exhibits the different orientation from the crystal structure. Global analysis of chemical shift perturbations found that increasing [NaCl] from 10 to 100 mM reduced the binding affinity of caZPKZ for both B-DNA (600-fold) and Z-DNA (25-fold) and decreased its B–Z transition activity (4.6-fold). Our results suggest that the structure of the intermediate complex formed by caZPKZ and B-DNA is modulated by varying salt concentration and thus it could be used as a molecular ruler to determine the degree of B–Z transition.

**ACCESSION NUMBERS**
The atomic coordinates have been deposited in the Protein Data Bank (PDB ID: 2RVC). Chemical-shift assignments have been deposited in the Biological Magnetic Resonance Bank (BMRM ID: 11595).

**SUPPLEMENTARY DATA**
Supplementary Data are available at NAR Online.

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