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Crystallization and preliminary X-ray crystallographic studies of the Z-DNA-binding domain of a PKR-like kinase (PKZ) in complex with Z-DNA

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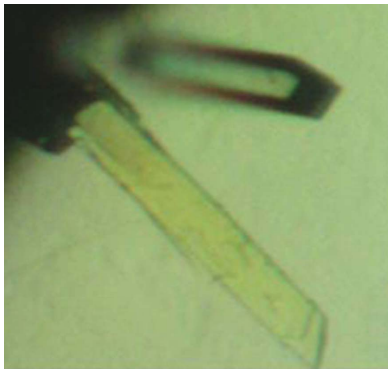
PKZ, a PKR-like eIF2α kinase, consists of a Z-DNA-specific binding domain (Zα) and an eIF2α kinase domain. The kinase activity of PKZ is modulated by the binding of Zα to Z-DNA. The mechanisms underlying Z-DNA binding and the subsequent stimulation of PKZ raise intriguing questions. Interestingly, the Z-DNA-binding domain of PKZ from goldfish (*Carassius auratus*; ca$Z\alpha_{PKZ}$) shows limited sequence homology to other canonical Zα domains, suggesting that it may have a distinct Z-DNA-recognition mode. In this study, the Z-DNA-binding activity and stoichiometry of ca$Z\alpha_{PKZ}$ were confirmed using circular dichroism (CD). In addition, preliminary X-ray studies of the ca$Z\alpha_{PKZ}$–Z-DNA complex are reported as the first step in the determination of its three-dimensional structure. Bacterially expressed recombinant ca$Z\alpha_{PKZ}$ was purified and crystallized with Z-DNA at 295 K using the microbatch method. X-ray diffraction data were collected to 1.7 Å resolution with an R_{merge} of 7.4%. The crystals belonged to the monoclinic space group $C2$, with unit-cell parameters a = 55.54, b = 49.93, c = 29.44 Å, β = 96.5°. Structural analysis of ca$Z\alpha_{PKZ}$–Z-DNA will reveal the binding mode of ca$Z\alpha_{PKZ}$ to Z-DNA and its relevance to other Z-DNA-binding proteins.

1. Introduction

The downregulation of protein synthesis *via* phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF2α) is a well established antiviral and antiproliferation mechanism (Proud, 2005). Double-stranded RNA (dsRNA) dependent protein kinase (PKR) is one of four kinases which mediate this phosphorylation in humans (Garcia *et al.*, 2007). PKR, as well as other known eIF2α kinases, is composed of a conserved kinase domain at the C-terminus and a regulatory domain at the N-terminus. The latter plays a role in the recognition of various environmental signals and the activation of the kinase domain. The phosphorylation activity of PKR is stimulated when it is bound to double-stranded RNA through the RNA-binding domain. Recently, interferon-inducible PKR-like kinases (PKZs) have been identified in fish species (Hu *et al.*, 2004; Rothenburg *et al.*, 2005; Su *et al.*, 2008). The kinase domain of PKZ is highly homologous to that of PKR and it shares common substrates with PKR (Bergan *et al.*, 2008). However, PKZ contains two Z-DNA-binding domains in its N-terminus instead of the dsRNA-binding domain (dsRBD) of PKR and accordingly modulation of its kinase activity is dependent on Z-DNA binding (Bergan *et al.*, 2008). Left-handed Z-DNA is a higher energy conformation of DNA which is generated as a consequence of negative supercoiling (Liu & Wang, 1987; Herbert & Rich, 1999). It has also been reported that the formation of Z-DNA owing to frequent mutations is associated with various genetic diseases (Wang *et al.*, 2006). Zα domains, which have been identified in dsRNA-editing enzyme ADAR1 (Herbert & Rich, 2001), DNA-dependent activator of interferon regulatory factors DAI (Schwartz *et al.*, 2001; Takaoka *et al.*, 2007) and poxvirus virulence factor E3L (Kim *et al.*, 2003), specifically bind to double-stranded nucleotides in left-handed conformations (Rich & Zhang, 2003).



The crystal structures of Zα domains in complex with Z-DNA have been reported for Z$\alpha_{\rm ADAR1}$ (Schwartz *et al.*, 1999), Z$\alpha_{\rm DAI}$ (Schwartz *et al.*, 2001) and Z$\alpha_{\rm E3L}$ (Ha *et al.*, 2004) and they revealed a common typical winged-helix motif. The recognition of Z-DNA is mediated by the recognition of phosphate backbones in zigzag conformation and the *syn* conformation of guanine. The residues present in the α3 helix and the β-wing region play important roles in Z-DNA recognition as they are involved in direct contacts with phosphate backbones as well as in water-mediated interactions (Schwartz *et al.*, 1999). Furthermore, structural analyses and mutational biochemical studies confirm that minor variations in the sequence of the β-wing region can alter the van der Waals interactions (Schwartz *et al.*, 2001) and Z-DNA stabilization (Quyen *et al.*, 2007). It was found that the Zα domain of PKZ from goldfish (*Carassius auratus*; caZ$\alpha_{\rm PKZ}$) shares limited sequence homology to other canonical Z-DNA-binding domains. 26% identity is observed between caZ$\alpha_{\rm PKZ}$ and hZ$\alpha_{\rm ADAR1}$ when 75 residues are compared. Interestingly, the Lys170 residue which plays a critical role in Z-DNA recognition is not present in caZ$\alpha_{\rm PKZ}$. Thus, very intriguing questions arise of how caZ$\alpha_{\rm PKZ}$ recognizes Z-DNA and consequently how Z-DNA binding modulates the kinase activity of PKZ. Structural study of caZ$\alpha_{\rm PKZ}$ is required to address these questions and here we present a preliminary crystallographic analysis of caZ$\alpha_{\rm PKZ}$ in complex with Z-DNA as the first step in the determination of its three-dimensional structure.

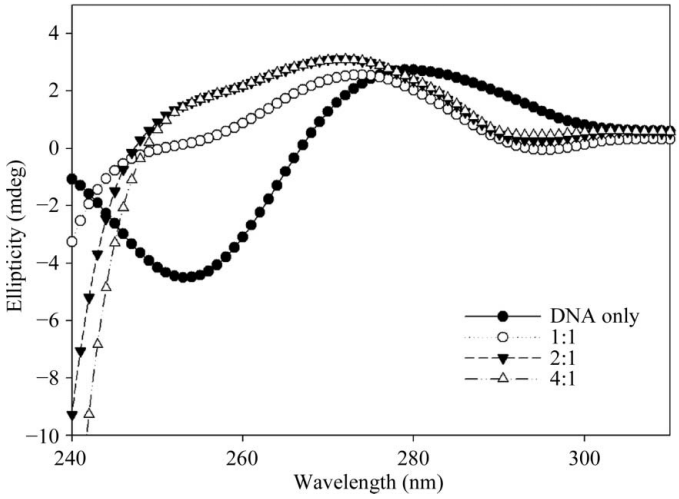


Figure 1
The B-DNA to Z-DNA conversion activity of caZ$\alpha_{\rm PKZ}$ measured by circular dichroism (CD). caZ$\alpha_{\rm PKZ}$ was added to d(TCGCGCG)$_2$ at the indicated molar ratios of 1:1, 2:1 and 4:1 and incubated for 1 h prior to the CD measurement. In the absence of caZ$\alpha_{\rm PKZ}$, the B-DNA spectrum was observed. The conformational change to Z-DNA was monitored by CD spectra between 240 and 310 nm.

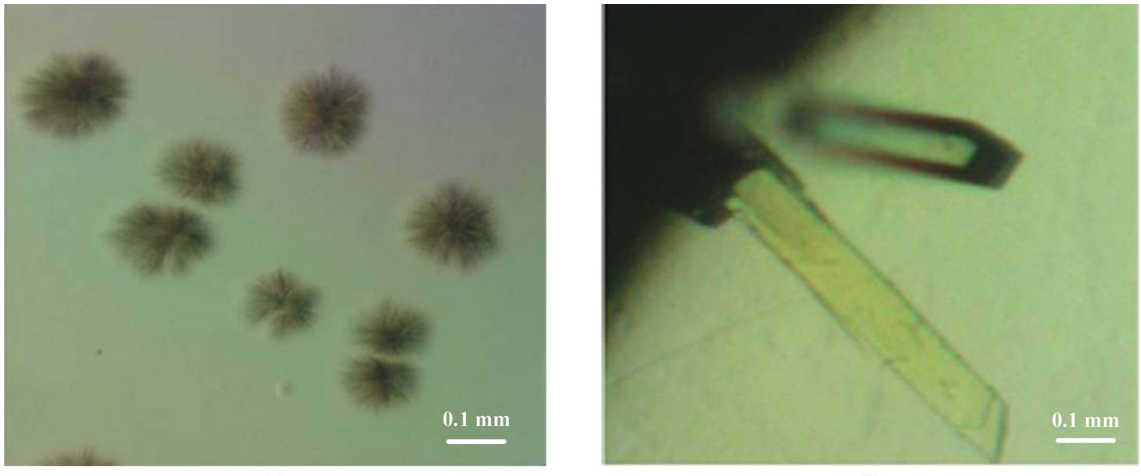
2. Materials and methods

2.1. Cloning, expression and purification

The coding sequence of the Zα domain (residues 1–75) of PKZ from *C. auratus* was cloned into pET28a+ (Novagen, Madison, Wisconsin, USA). As a result, an extra six histidine residues were attached to the N-terminus of caZ$\alpha_{\rm PKZ}$ and were subsequently removed during purification. *Escherichia coli* BL21(DE3) cells (Novagen, Madison, Wisconsin, USA) transformed with this recombinant plasmid were grown in Luria–Bertani medium containing 30 mg ml^{-1} kanamycin at 310 K and 0.1 m*M* isopropyl β-D-1-thiogalactoside (IPTG) was added when the OD$_{600}$ reached 0.6. The cells were harvested after 3 h and caZ$\alpha_{\rm PKZ}$ was purified essentially as described elsewhere (Schwartz *et al.*, 1999). Briefly, after initial chromatography on a HiTrap metal-chelating column (GE Healthcare, Princeton, New Jersey, USA), thrombin was added to remove the hexahistidine tag and caZ$\alpha_{\rm PKZ}$ was further purified using a Resource S ion-exchange column (GE Healthcare, Princeton, New Jersey, USA). The purified caZ$\alpha_{\rm PKZ}$ was dialyzed against buffer *A* (5 m*M* HEPES pH 7.5 and 10 m*M* NaCl) and concentrated to 1 m*M*. The protein concentration was determined using the Bradford method.

2.2. Preparation of the double-stranded DNA

The double-stranded DNA (dsDNA) was obtained by heating the single-strand oligomer d(TCGCGCG) (Bioneer, Daejeon, Korea) in buffer *A* for 10 min at 353 K followed by gradual cooling to 277 K. dsDNA was then isolated by MonoQ ion-exchange column chromatography (GE Healthcare, Princeton, New Jersey), dialyzed against distilled water and lyophilized for storage and crystallization. The DNA concentration was calculated by UV spectroscopy.



(*a*) (*b*)

Figure 2
Crystals of the caZ$\alpha_{\rm PKZ}$–d(TCGCGCG)$_2$ complex. (*a*) Crystals obtained in the initial screening. (*b*) Diffraction-quality crystals obtained using the optimized crystallization conditions. The approximate dimensions of the optimized crystals are 0.1 × 0.1 × 0.5 mm.

2.3. Z-DNA conversion assay

The Z-DNA conversion activity of caZα_{PKZ} was measured by circular dichroism (CD) using 30 µ*M* d(TCGCGCG)$_2$ in buffer *A*. CD spectra were obtained at 298 K using a Jasco J-810 CD spectrometer (Jasco, Tokyo, Japan) and a 0.1 cm quartz cell. The volume of the concentrated caZα_{PKZ} stock solution added to each reaction did not exceed 5% of the total volume. The mixture was equilibrated for 1 h prior to measurement. Spectra were recorded between 240 and 310 nm at 1 nm intervals averaged over 2 s.

2.4. Crystallization

Purified caZα_{PKZ} protein (0.6 m*M*) was mixed with dsDNA in a 1:2 molar ratio in buffer *A* and incubated at 303 K for 2 h. Initial crystallization screening was performed manually at 295 K by the microbatch method using Crystal Screens I and II (Hampton Research, Aliso Viejo, California, USA) and Cryo I and II kits (Emerald Biosystems, Bainbridge Island, Washington, USA). Each drop consisting of 1 µl screen solution and 1 µl protein–DNA mixture was covered with 10 µl Al's Oil (Hampton Research, Aliso Viejo, California) in Nunclon Δ Surface wells (Nunc, Rochester, New York, USA). In order to optimize the initial crystallization conditions, a number of parameters were varied.

2.5. Data collection

Complete diffraction data were collected using a MAR CCD 165 mm detector on the BL4A beamline of the Pohang Accelerator Laboratory Synchrotron (Pohang, Korea) from a crystal that was flash-cooled in a cold nitrogen-gas stream at 100 K. Prior to data collection, the crystal was immersed for more than 30 s in mother liquor (30% PEG 1500, 15 m*M* $MnCl_2$) containing 25%(*v*/*v*) glycerol as a cryoprotectant. The wavelength of the synchrotron radiation was 1.000 Å. The diffraction data were processed and scaled using *HKL*-2000 (Otwinowski & Minor, 1997).

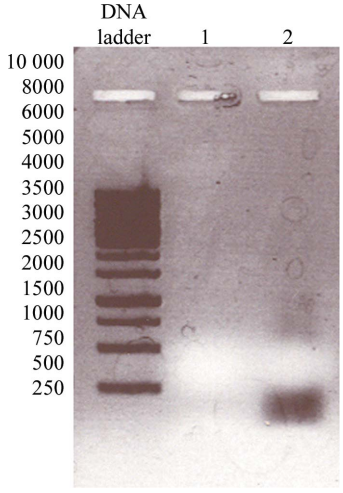


Figure 3
Crystals of the caZα_{PKZ}–d(TCGCGCG)$_2$ complex dissolved in 10 µl distilled water were loaded onto a 0.8% agarose gel and stained with EtBr. Lane 1, which contained only protein, did not stain with EtBr. Lane 2, which contained the dissolved crystals, picked up the dye, demonstrating that the crystals contained the protein–DNA complex.

Table 1
X-ray data-collection and processing statistics.

Values in parentheses are for the last shell.

Wavelength (Å)	1.000
Space group	*C*2
Unit-cell parameters (Å, °)	a = 55.54, b = 49.93, c = 29.44, β = 96.5
Resolution (Å)	20.0–1.70 (1.76–1.70)
Unique reflections	29661 (8586)
Completeness (%)	96.5 (90.3)
Redundancy	1.9
R_{merge}† (%)	7.4 (15.4)
$\langle I/\sigma(I)\rangle$	35.9 (7.3)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

3. Results and discussion

Zα_{PKZ} from goldfish was expressed in *E. coli* and purified for structural studies. Approximately 10 mg of homogenous protein was obtained per litre of culture. The CD spectrum of the caZα_{PKZ}–d(TCGCGCG)$_2$ mixture indicated a typical Z conformation of the DNA, which confirmed that caZα_{PKZ} is able to convert d(TCGCGCG)$_2$ to the Z conformation. The CD spectra showing the Z-DNA conformation were virtually the same when caZα_{PKZ} was added to DNA in a twofold or fourfold molar excess, suggesting that the formation of Z-DNA in the mixture was saturated at a caZα_{PKZ}:d(TCGCGCG)$_2$ ratio of 2:1 (Fig. 1). This finding is consistent with previous structure studies (Schwartz *et al.*, 1999, 2001; Ha *et al.*, 2004) showing that a Z-DNA-binding protein binds to each strand of d(TCGCGCG)$_2$ in the Z conformation.

In the initial crystallization screen, clusters of needle-shaped crystals of the caZα_{PKZ}–Z-DNA complex were obtained using condition No. 43 (30% PEG1500) from Crystal Screen I (Hampton Research, Aliso Viejo, USA; Fig. 2*a*). However, the shape and size of these crystals were not suitable for X-ray diffraction experiments. The addition of $MnCl_2$ resulted in a change in crystal shape from clusters of needles to tetragonal columns (Fig. 2*b*) and diffraction-quality crystals were finally observed using 30% PEG 1500 and 15 m*M* $MnCl_2$ at 295 K. The crystals grew to final dimensions of 0.1 × 0.1 × 0.5 mm within 2 d (Fig. 2*b*). The presence of DNA in the crystal was confirmed by EtBr staining of the dissolved crystals on 0.8% agarose gel (Fig. 3).

X-ray diffraction data were collected from a cryoprotected crystal to 96.5% completeness at 1.7 Å resolution with an R_{merge} of 7.4%. The crystal belonged to the monoclinic space group *C*2, with unit-cell parameters a = 55.54, b = 49.93, c = 29.44 Å, β = 96.5°. Assuming the presence of one caZα_{PKZ} molecule and one single-stranded DNA molecule per asymmetric unit, the Matthews coefficient V_M was calculated to be 2.01 Å^3 Da^{-1}, which corresponds to 45.4% solvent content. This V_M value is within the range commonly observed for protein crystals (Matthews, 1968). The data-collection and processing statistics are summarized in Table 1. We intend to solve the structure of caZα_{PKZ} complexed with dsDNA by molecular replacement using the Zα domain of human ADAR1 bound to d(TCGCGCG)$_2$ (PDB code 1qbj) as a template model.

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