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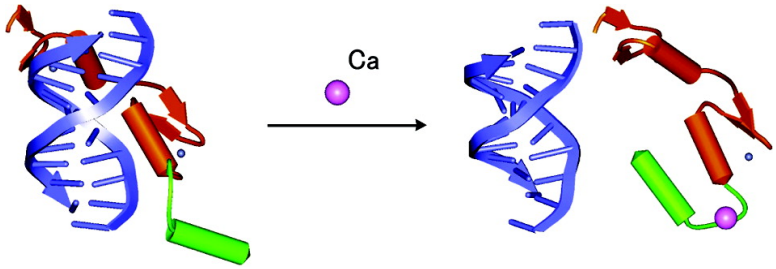

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Calcium Ion Responsive DNA Binding in a Zinc Finger Fusion Protein

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Abstract: Zinc finger fusion proteins, having a Ca-binding site from troponin C, were created to develop Ca-responsive regulation of DNA binding. The typical zinc finger folding of a novel fusion protein with a single finger, F2-Tn, was investigated using UV−vis spectroscopy of the Co-substituted form and CD experiments. Detailed structural analyses of F2-Tn/Zn^{2+} using NMR experiments and structural calculations clarify that our fusion protein gives a native zinc finger folding with the artificial Ca-binding domain intervening two helices. The Ca-responsive DNA-binding affinity of troponin-fused protein with two fingers (using F1F2-Tn) was investigated by electrophoretic mobility shift assay (EMSA). EMSA analyses of F1F2-Tn were performed under the conditions of various concentrations of the Ca ion. F1F2-Tn has a K_d value of 5.8 nM in the absence of Ca ion and shows a higher K_d value of 13 nM in the presence of 100 equiv of Ca ion. The artificially designed fusion zinc finger protein with a Ca-binding domain has Ca-responsive DNA-binding affinity. It is leading to a better understanding of the construction of zinc finger-based artificial transcriptional factors with a Ca switch.

Introduction

The use of zinc fingers is an attractive strategy for the design of DNA-binding proteins with sequence specificity. Controlling the binding availability by further improvement on zinc fingers is a promising method for the regulation of gene expression. The Cys_2His_2 type of zinc finger is the most frequent motif and folds into a $\beta\beta\alpha$ structure in the presence of Zn^{2+}.[1,2] Each finger structure recognizes a sequential three base pair subsite of DNA, and the connection of fingers realizes the recognition of longer unique DNA base pairs. The four key amino acid residues in the helix of the finger unit contribute to the specific DNA binding[3−8] The creation of zinc finger variants with novel DNA-binding specificities was established with designed modifications, such as the mutagenesis on key residues, or domain swapping,[9] and phage display strategy.[10−17] Some of the artificial fingers successfully led to manipulating gene expression in vivo.[18−21]

Zinc finger domains are frequently found as a part of various kinds of transcriptional factors, and the DNA-binding ability of the zinc finger region can be regulated by the other part of the protein. Thus, artificial transcriptional factors with switching functions have recently been reported with zinc finger fusion proteins or their mutants. Artificial fusion of a zinc finger with a steroid hormone receptor was reported as a chemically regulated transcriptional factor.[22] A designed dimeric zinc finger was created that binds cooperatively to DNA via an attached leucine zipper dimerization domain.[23] Small-molecule switches

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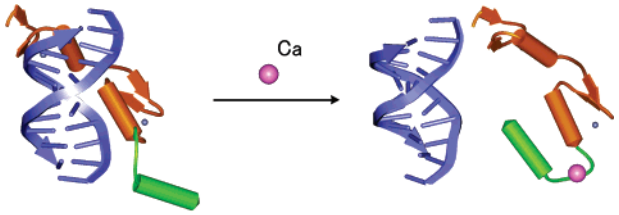

Figure 1. Strategy of Ca-sensing fusion protein with zinc finger domain (orange) and an additional Ca-binding site from troponin C (green).

for zinc fingers have been established with mutations on significant amino acid residues for DNA binding.[24]

We were motivated to create artificial zinc finger fusion proteins in order to develop the metal-responsive regulation of DNA binding (Figure 1). Several DNA-binding proteins containing a metal-binding domain are known to play a crucial role in maintaining metal homeostasis.[25] Fur (ferric uptake regulator) protein, when bound by Fe^{2+} ion, binds its target DNA within the promoter of the regulated gene to repress transcription.[26] The MerR metalloregulatory protein functions as a repressor and Hg^{2+}-responsive transcription activator.[27] DREAM (Downstream Regulatory Element Antagonist Modulator) works as Ca-dependent repressor containing EF-hand motif.[28] However, zinc finger proteins that sense other metals and the control of their DNA binding have not been reported. We have searched for reported zinc finger proteins that have an additional metal-binding domain with BLAST and MOTIF database and found the MET31_YEAST gene coding zinc finger proteins with a neighboring Ca-binding motif similar to that of calmodulin.[29] Diacylgrycerol kinase has two sets of EF-hand motifs and two sets of cysteine-rich zinc fingers.[30] The family of Ca-binding proteins, such as troponin C,[31−33] calmodulin,[34,35] and parvalbumin,[36,37] have a characteristic structural transition from the apo to the calcium-bound form with the EF-hand motif consisting of a helix−loop−helix.[38] These structural changes work as a Ca switch in various families of proteins having the EF-hand motif. Thus, we expect the existence of some unknown proteins containing both the zinc finger and Ca-binding domain, which works as a Ca-dependent transcriptional factor. Otherwise, we artificially construct such proteins, express them in the cell, and we will join directly the Ca homeostasis and the expression of the target gene using zinc finger-based transcriptional factors. Thus, we have designed a Ca-responsive finger that is a fusion protein with a representative EF-hand motif of a Ca-binding protein in order to control DNA binding (Figure 1). Our artificial zinc finger fusion proteins are composed of zif268 finger2 and a loop−helix region of troponin C. Herein we report the characterization of our fusion proteins with single finger, F2-Tn, and the Ca-dependent DNA-binding affinity with two fingers, F1F2-Tn, to develop the fundamental strategy for creating zinc finger-based artificial transcriptional factors with a Ca switch.

Results and Discussion

Zinc Finger Fusion Protein. The amino acid sequence of novel zinc finger proteins with Ca-binding domain, F2-Tn and F1F2-Tn, is shown in Figure 2. Each domain of the zinc finger gives $\beta\beta\alpha$ folding, and troponin C has a typical helix−loop−helix structure, named the EF-hand motif, as the Ca ion binding site. We have connected the C-terminus of the helix from the zinc finger with the loop−helix part of the EF-hand motif to create an additional Ca-binding region. The length of amino acid residues in the connecting linker region was designed using molecular dynamics simulation to form the favorable EF-hand structure.[39] The amino acid sequence of the finger domain was modified from a native protein, finger 2 of zif268, to give a stronger DNA-binding affinity. Amino acid residues in positions −1, 2, 3, and 6 of the helix define the recognition sequence of DNA. We have designed the amino acid sequence of F1F2-Tn to bind to the 5′-GGGGCG-3′ region of DNA in accordance with the previously reported recognition code.[13,40,41] Some of the residues were changed to arginine residues in which position the other native finger has arginine residues. The methionine residue was swapped for glycine to prevent oxidation. Each finger was connected with a typical canonical linker, TGEKP, which supports the stronger DNA-binding affinity.

F2-Tn and F1F2-Tn were synthesized by the combination of solid phase and native chemical ligation methods.[42−44] The final products were purified by reverse phase HPLC and analyzed by MALDI-TOF mass spectroscopy.

The Zn-binding ability of our fusion protein was investigated in ESI-TOF MS experiments. Zinc-bound F2-Tn/Zn was significantly detected (m/z = 1331.6; calcd for [M + Zn^{2+} + 2 H^+]/4: m/z = 1332.7 and m/z = 1078.5. Calcd for [M + Zn^{2+} + 3H^+]/5: m/z = 1079.1).

Cys_2His_2 Coordination and Folding Structure. The coordination environment of the Cys_2His_2 moiety was investigated by Co^{2+} titration using absorption spectroscopy. The Co^{2+}-loaded form of the F2-Tn showed absorption maxima at 565 and 645 nm (Figure 3a), which is consistent with tetrahedral Cys_2His_2/Co^{2+} coordination in zinc fingers.[45] The titration

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(39) The molecular dynamics simulation in vacuum was performed using the AMBER6 program. The model of ZF-Tn with several lengths of the linker and the target dsDNA structure was constructed based on the reported ZF-DNA structures. The structure after 10 ps of MD simulation was evaluated for the design of the linker sequence.
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Zif268 FQCRICMRNFSRSDHLTTHIRTHTG

F2-Tn YQCRICGRNFSRSDHLTRHIRTHTGDKNNDGRIDAEEALEILRAKG

F1F2-Tn CRICGRNFSRSDDLTRHIRTHTGEKP-
YGCRICGRNFSRSDHLTRHIRTHTGDKNNDGRIDAEEALEILRAKG

Troponin C DKNADGFIDIEELGEILRATG

Figure 2. Primary sequences of novel zinc fingers F2-Tn and F1F2-Tn. The amino acid sequence of native zinc finger (zif268, finger 2) and chicken skeletal troponin C are also listed.

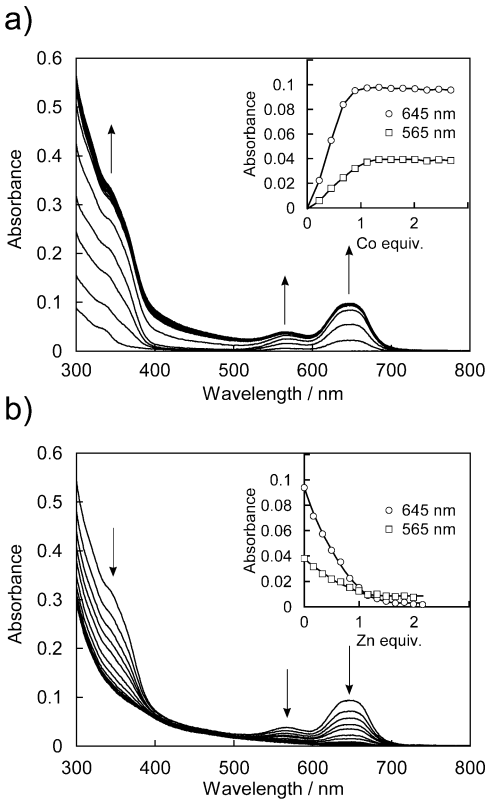

Figure 3. (a) Co^{2+} titration of apo F2-Tn protein using UV−vis spectroscopy (0.2 mM F2-Tn, 50 mM HEPES (pH 7), 0.1 M NaCl, 0.1 M $CoSO_4$ solution). (b) Zn^{2+} reverse titration of F2-Tn/Co (0.2 mM F2-Tn/Co, 50 mM HEPES (pH 7), 0.1 M NaCl, 0.1 M $ZnSO_4$ solution). Insets: titration curve of each experiment.

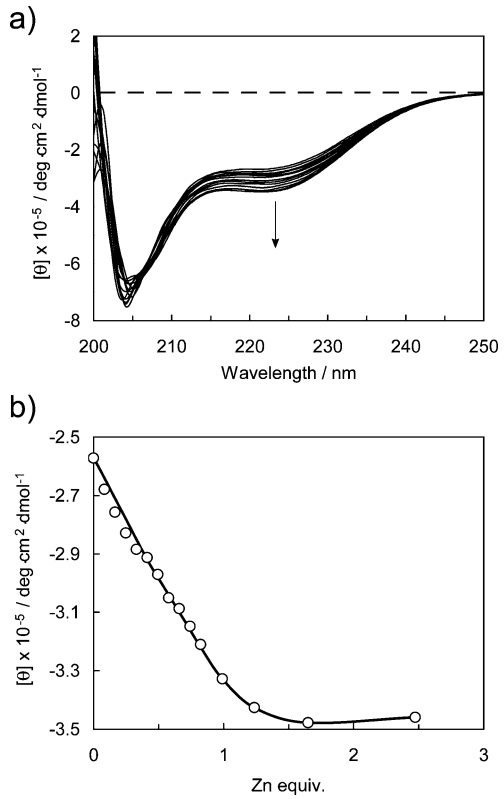

Figure 4. Titration of F2-Tn/Zn with Zn^{2+} using CD spectroscopy. Sample solution was 0.1 mM F2-Tn, 20 mM HEPES (pH 7), 0.1M NaCl, 0.1 M $ZnSO_4$ solution.

experiments of adding Co^{2+} clearly suggest that F2-Tn binds to 1 equiv of Co^{2+}. In the reverse titration experiment, adding Zn^{2+} to the F2-Tn/Co, the aforementioned transition bands assigned as Cys_2His_2/Co^{2+} coordination decreased, indicating that Co^{2+} was substituted for Zn^{2+} (Figure 3b). The titration curve of the reverse experiments also supports the idea that F2-Tn binds to 1 equiv of Zn^{2+} ion. Thus, Zn^{2+} does not interact with the additional Ca-binding site in our fusion protein.

The folding structure of the Zn^{2+}-bound form, F2-Tn/Zn, was investigated by CD spectroscopy. The Cys_2His_2 zinc finger exhibits characteristic negative extrema at about 206 and 222 nm, due to the ordered secondary structure containing an α-helix (the apo form does not show such extrema).[46−48] Our apo form of F2-Tn gives a negative band at 222 nm, supporting the idea that the troponin helix already exists in the absence of Zn^{2+} as shown in Figure 4a. The negative extremum based on helix formation increased with the addition of Zn^{2+} up to 1 equiv (Figure 4b). These results suggest that the F2-Tn peptide with additional Ca-binding domain folds into the native zinc finger in the presence of Zn^{2+}.

Complexation of apo F2-Tn with Zn^{2+} was also pursued by competition experiment against Co^{2+}, using CD spectroscopy. Co-loaded F2-Tn exhibits positive and negative extrema between 250 and 400 nm, as shown in Figure 5a. The intensities of observed extrema are decreased upon addition of Zn^{2+}. The series of titration curves at each extremum also indicate a 1:1 complexation between peptide and Zn^{2+} ion (Figure 5b).

Solution Structure. Detailed structural analyses of F2-Tn/Zn using NMR experiments clarify that our fusion protein with the artificial Ca-binding domain gives a folding similar to that of the native zinc finger. 2D NMR experiments were performed in the presence of 1.5 equiv of Zn^{2+} in 90% H_2O/10% D_2O (deuterated Tris buffer) (Figure 6). Proton resonances of F2-Tn were assigned by following the conventional strategy of spin-system assembly and sequential assignment, using standard homonuclear 2D NMR spectra.[49] The obtained ROE connectivities are shown in Figure 7. Two significant stretches of $d_{\alpha N}$-

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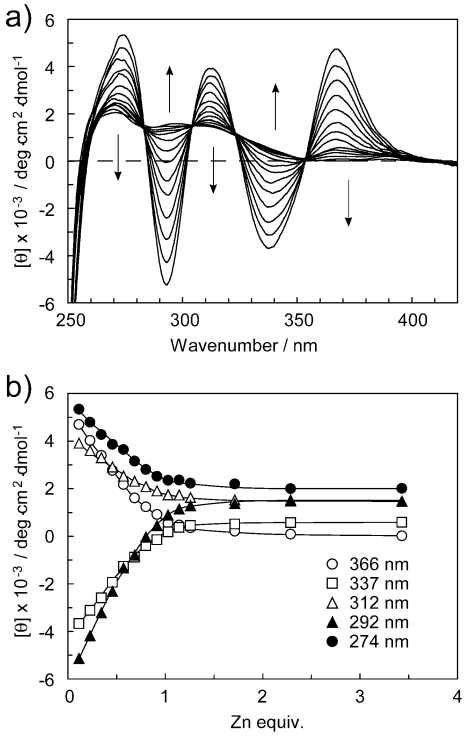

Figure 5. Titration of F2-Tn/Zn with Zn^{2+} using CD spectroscopy. Sample solution was 0.1 mM F2-Tn, 20 mM HEPES (pH 7), 0.1M NaCl, 0.1 M $ZnSO_4$ solution.

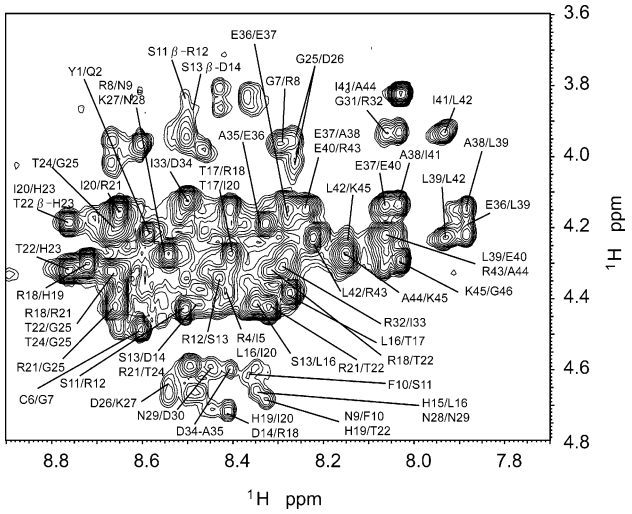

Figure 6. NH−C_αH region of ROESY spectra of 2 mM F2-Tn and 3 mM $ZnSO_4$ in 10% D_2O/90% H_2O (deuterated Tris buffer, pH 6) at 7 °C; 600 MHz; mixing time, 200 ms.

($i,i+3$) and $d_{\alpha N}(i,i+4)$ connectivities for residues 13−23 and 35−46 suggest that helical conformation is present in these regions (Figure 7). The three-dimensional structure of the protein was computed from ROE and $^3J_{HNH\alpha}$ restraints by the simulated annealing method[50] with the XPLOR program. Superimposed NMR structures of the zinc finger domain and the Ca-binding loop−helix domain of F2-Tn are illustrated in Figure 8, parts a and b, respectively. The ribbon representation of ZF-Tn is shown in Figure 8c. The finger domain folds in to the $\beta\beta\alpha$ structure, which is similar to that of native proteins. The C-terminus Ca-binding domain gives a helix structure as suggested by CD experiments. The intervening loop for Ca binding is flexible in the absence of the Ca ion. The NMR results indicate that our fusion zinc finger protein actually has a native finger structure and the helix−loop−helix motif for the Ca-binding site is as we designed it.

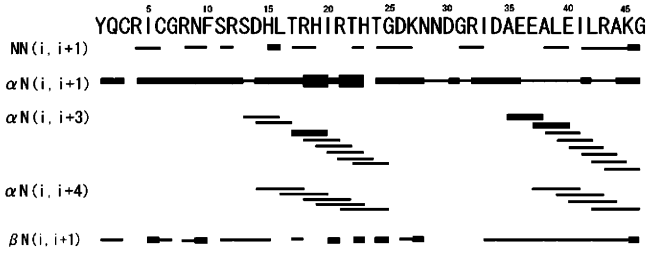

Figure 7. Summary of sequential and medium-range ROE connectivities for the Zn-bound F2-Tn. The thickness of the bars is a qualitative measure of cross-peak intensity in a ROESY spectrum.

We checked the F2-Tn conformation when Ca ion is loaded into the binding site. In the CD experiment, the characteristic negative extremum (204 and 222 nm) observed in F2-Tn/Zn did not change in the presence of 1 and 5 equiv of Ca ion. Therefore, the α-helix structure of the fusion zinc finger is preserved. A slight change around 205 nm presumably indicates the conformational rearrangement of the loop region upon Ca binding.

DNA-Binding Assay. With the use of F1F2-Tn/Zn, the Ca-responsive DNA-binding affinity of troponin C-fused protein with two fingers was investigated by electrophoretic mobility shift assay (EMSA). The finger domains, F1 and F2, were designed to bind to GGG and GCG, respectively, according to the reported recognition code. The experiments for K_d measurements were performed using a 50 bp DNA duplex, which contained a 5′-GGGGCG-3′ fragment. It was confirmed that the protein actually binds to the target DNA subsite by performing a binding assay in the presence of the DNA probe without a recognition sequence. It is anticipated that a small amount of EDTA contained in the DNA solution will inhibit Ca binding to the fusion protein. Thus, EDTA molecules were carefully removed from solution by gel filtration and dialysis.[51] Figure 9 shows EMSA results of F1F2-Tn/Zn in the absence and in the presence of Ca ion. F1F2-Tn/Zn has a K_d value of 5.8 nM in the absence of the Ca ion and 13 nM in the presence of 100 equiv of Ca ion. The K_d values gradually increased to roughly twice the higher value (Figure 10). However, F1F2/Zn (without a Tn Ca-binding site) does not exhibit such an increase of K_d. Therefore, the results clearly indicate that the fusion zinc finger has potential for Ca-responsive DNA-binding properties.

The decrease of DNA-binding affinity for Ca-bound ZF-Tn is thought to occur as a result of the conformational change of the Ca-binding loop. The α-helical domain of the zinc finger protein binds to a major groove of the DNA, and the amino acid residues in the α-helical domain recognize the base pair. The resulting loop domain is flexible and does not interfere with the DNA binding in the absence of Ca ion, while the loop region containing the Ca ion obtains a fixed conformation that causes a steric hindrance for binding with the DNA molecule. Thus, Ca-bound ZF-Tn has a lowered DNA-binding affinity. The small

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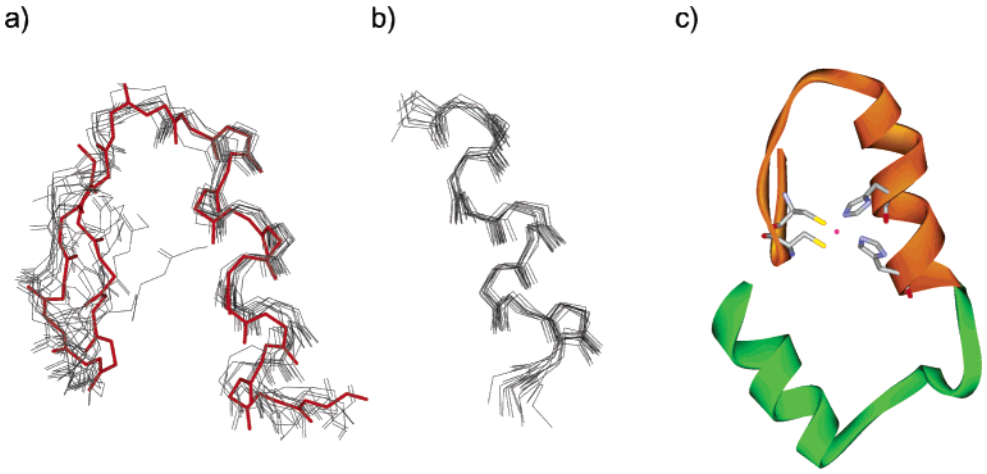

Figure 8. Superimpositions of the backbone atoms of the 10 NMR structures of (a) zinc finger domain (1−26) and (b) helix domain of troponin C (35−46), best fitted to the helix part. (Red stick shows native finger structure.) (c) Overall structure of zinc-bound F2-Tn in ribbon representation.

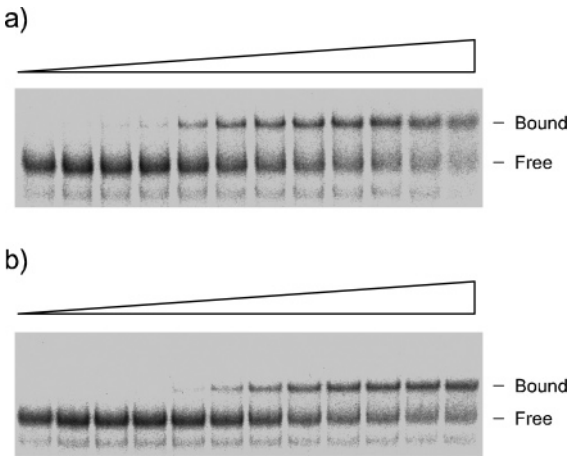

Figure 9. EMSA analyses of DNA binding of Zn-bound F1F2-Tn (a) in the absence and (b) in the presence of 100 equiv of Ca^{2+}. Concentration of F1F2-Tn was increased from left to right lane (0, 0.24, 0.49, 0.98, 1.95, 3.91, 7.81, 15.6, 31.3, 62.5, 125, 250 nM). Each loading mixture contained 10 mM Tris-HCl (pH 8), 33.3 mM NaCl, 5% glycerol, 0.005% NP-40, 1 mM DTT, ^{32}P-end-labeled substrate DNA fragment (ca 500 cpm), poly-(dI-dC) (10 ng/μL), a selected concentration of zinc finger peptides, and 3 equiv of $ZnSO_4$.

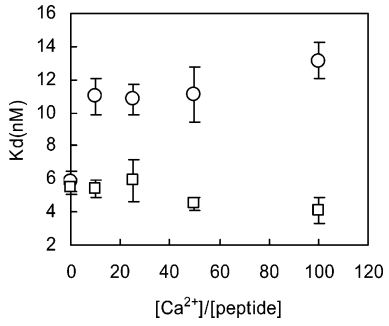

Figure 10. Ca ion dependence of K_d values for F1F2-Tn/Zn (circles) and F1F2/Zn (squares) with standard deviations.

difference in the K_d values between the two states, Ca-bound and Ca-free, has a relationship with the Ca-binding affinity. The helix in the N-terminus of the helix−loop−helix motif contains hydrophobic residues, which seem to have an important influence on the interaction between the two helices in stabilizing the Ca-bound EF-hand structure. In our fusion protein, the helix in the zinc finger domain has many hydrophilic residues to prevent such interaction, which leads to a lowering the Ca-binding affinity.

Conclusions

We have developed a novel zinc finger protein with a Ca-binding loop of troponin C for which structural analyses indicate that the fusion protein folds into a native zinc finger structure with a helix−loop−helix Ca-binding domain. Our fusion protein with a two-finger unit has DNA-binding affinity, which is controlled by the coordination of a Ca ion. The results indicate that the conformational change upon binding of Ca ion decreases the binding affinity between the DNA recognition region of the protein and the target DNA molecule in our newly developed zinc finger protein. The switching potential as indicated by the K_d values in this protein is not still satisfactory, but further improvement in the Ca-binding affinity will enable a more specific response to Ca ion signals. The successful strategy for gene regulation using the zinc finger fusion proteins having a transcriptional activator or repressor domains was reported[52] in the literature. In conjunction with this, the creation of a zinc finger and troponin C fusion will be a possible new strategy for a gene regulation system with a Ca switch.

Experimental Section

Physical Measurements. All spectroscopic experiments were performed under nitrogen atmosphere to avoid oxidation of cysteine residues. UV−vis spectra were recorded on a Shimadzu spectrophotometer UV2200A. Circular dichroism (CD) spectra were measured on a Jasco J-725. MALDI-TOF MS analyses were performed using a PerSpective Biosystems Voyager LinerRD VDA-500. ESI-TOF MS analyses were performed using a Micromass LCT and Bruker microTOF.

Solid Phase Peptide Syntheses. Peptides were manually synthesized by standard solid-phase methods with Fmoc chemistry with *N*,*N*-diisopropylcarbodiimide (DIC)/hydroxybenzotriazole (HOBt) or benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP)/HOBt activation and native chemical ligation methods. H-Gly-2-ClTrt resin was used to synthesize peptides used in this study. The unprotected peptides were cleaved form the resin by treatment with a solution of 90% TFA, 4% 1,2-ethanedithiol, 4% H_2O, 2% triisopropylsilane. The peptides were washed five times with cold ether to remove any remaining scavengers. Protected peptides were cleaved by

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treatment with 80% DCM, 10% AcOH, 10% TFE. C-terminal thioester peptides were synthesized by coupling of protected peptide fragments and 3-mercaptopropionic acid by PyBOP/HOBt activation. Native chemical ligation was performed with the following: 0.1 M sodium phosphate buffer (pH 8.0) containing 6 M guanidin hydrochloride, 4% (vol/vol) thiophenol, 4% (vol/vol) benzylmercaptan. All peptides were purified on a C18 reverse phase HPLC column with an acetonitrile gradient containing 0.05% TFA. All peptides were characterized by MALDI-TOF MS analyses.

NMR Spectroscopy. NMR experiments were performed with 2 mM F2-Tn peptide and 3 mM $ZnSO_4$ in 10% D_2O/90% H_2O (deuterium Tris buffer, pH 6). The most appropriate temperature for experiments was determined to be 7 °C. ^{1}H NMR spectra were acquired on a Varian Inova 600 MHz spectrometer. All complete proton resonance assignments were made using DQF-COSY, TOCSY,[53,54] NOESY, and ROESY[53,56] experiments. All spectra were recorded with 9000.9 Hz spectral width in both dimensions and 2048 data points in *f*2 and 512 data points in *f* 1 with eight scans at each increment. The water signal was attenuated using presaturation during the relaxation delay of 1.2 s.

All data obtained at 600 MHz were processed with NMRPipe and analyzed with PIPP software. Interproton distances were derived from the hononuclear 2D ROESY spectra recorded with a mixing time (τ_m) of 200 ms. Appropriate values for pseudo atoms were added to distance restraints involving methyl protons and nonstereospecifically assigned methylene protons. The ROE restraints were classified as strong, medium, weak, and very weak, corresponding to distance restraints of 1.8−3.0, 1.8−4.0, 2.2−5.0, and 3.2−5.9, respectively. These were characterized on the basis of the total volumes of the cross-peaks. The vicinal coupling constants ($^3J_{NH-H}{}^{\alpha}$) were measured by deconvolution of extracted rows using a line fitting with DQF-COSY spectra.

Molecular Modeling. The three-dimensional structure of the peptide was computed from the experimental restraints by using a simulated annealing method with XPLOR(version 3.851).[57] All calculations were carried out on a Linux system. The molecular dynamics calculation in vacuo started from an extended conformation, and the initial velocities of atoms in the molecular dynamics were randomly given. One hundred structures were generated and optimized against distances in the simulated annealing method described by Brünger, except the numbers of steps increased for the both the high-temperature and the cooling steps.[57] The 10 lowest energy accepted structures (no ROE violations of > 0.4 Å) were used for further structural consideration. The structure statistics are summarized in Table 1. Geometry of structures and structural parameters were analyzed using PROCHECK-NMR.[58]

Electromobility Shift Assay. Each reaction mixture contained 10 mM Tris-HCl (pH 8), 33.3 mM NaCl, 5% glycerol, 0.005% NP-40, 1 mM DTT, ^{32}P-end-labeled substrate DNA fragment (ca 500 cpm), and a selected concentration of zinc finger peptides. The sequence of target DNA is shown below.

5′-AGCTTAGCAGCTGA**GGGGCG**TAATAGTAGCTTTGAATA-GATTGAAT AGAT-3′

3′-ATCGTCGACT**CCCCGC**ATTATCATCGAAACTTATCTAA-CTTATCTAG ATC-5′

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Table 1. Restraints and Structural Statistics

distance restraints	474	
intraresidue	304	
sequential, $\|i-j\|=1$	110	
medium range, $1<\|i-j\|\le 4$	47	
long range, $\|i-j\|>4$	13	
dihedral angel restraints	33	
hydrogen-bond restraints	30	
NOE violations > 0.4 Å	0	
statistics for structural calculations		
rmsd from idealized covalent geometry		
bonds (Å)	0.0032 ± 0.0002	
angles (deg)	0.5504 ± 0.0237	
improper (deg)	0.3643 ± 0.0163	
rmsd from experimental data		
NOEs (Å)	0.0483 ± 0.0025	
dihedral (deg)	0.3973 ± 0.1530	
average ensemble rmsd (Å)	backbone atoms	heavy atoms
zinc finger domain (3−23)	0.73	1.46
troponin C domain (35−46)	0.43	1.26
Ramachandran analysis (%)[a]		
residues in most favored regions	68.0	
residues in additional allowed regions	27.3	
residues in generously allowed regions	4.1	
residues in disallowed regions	0.5	

[a] Generated using PROCHECK-NMR on the ensemble of the 10 lowest-energy structures.

Unincorporated ^{32}P label and EDTA were carefully removed by gel filtration equilibrated in deionized water, followed by dialysis against water, before use. The experiment was performed in the presence of a competitor DNA, poly(dI-dC) (10 ng/μL). The binding reaction mix was incubated at 25 °C for 30 min. Gels were preelectrophoresed for at least 2 h to constant current. The DNA-bound proteins and free probe were electrophoretically separated on a 20% polyacrylamide gel with 0.5× Tris-borate running buffer containing 100 mM $ZnSO_4$ at 180 V. The gels were vacuum-dried and visualized by autoradiography using Fuji BAS 2000. The bands were quantified with FUJI FILM Science Lab 2001 Image Gauge (version 4.0) software. The fractions labeled DNA bound to zinc finger peptides were calculated using the equation $R = I_b/(I_b + I_f)$, where I_b and I_f are the intensities of peptide-bound and free DNA bands, respectively. The K_d values were evaluated by fitting the experimentally obtained values of R to the binding isotherm equation $R = [\text{peptide}]/([\text{peptide}] + K_d)$ and averaged from three independent experiments. The data in the range of $0.1 \le R < 0.9$ were used for evaluation.

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Supporting Information Available: Experimental results of NMR experiments, CD spectroscopy, and complete ref 29. This material is available free of charge via the Internet at http://pubs.acs.org.

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