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Conversion of Antennapedia Homeodomain to Zinc Finger-like Domain: Zn(II)-Induced Change in Protein Conformation and DNA Binding

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Protein structural change plays an important or critical role in biologically related events such as the protein-folding process,[1] functional regulation (for example, serine protease inhibitor[2] and calmodulin[3]), and conformational disease.[2] In view of not only understanding such dynamic properties of proteins but also designing a functional protein, it is interesting to create an artificial protein which induces structural change and also regulates its function by a simple cofactor such as a metal ion. Thus far, considerable studies on the design of metalloproteins have been performed.[4] In most of them, the metal-binding site was constructed on a natural protein scaffold, using the fixed backbone model of the protein. However, the studies focused on protein structural change in the field of metalloprotein design are very sparse, although the designs of proteins inducing a domain motion[5] or unfolding[6] have been reported. Here, we successfully engineered a novel protein, "Antennafinger (Ant-F)", whose structure and function can be controlled through Zn(II) binding by introducing the consensus residues of a Cys_2His_2-type zinc finger motif[7] into a non-metalloprotein scaffold, an Antennapedia homeodomain mutant (Ant-wt) (Figures 1 and 2).[8]

In the first step of the design procedure, the consensus residues of the Cys_2His_2-type zinc finger were employed as a metal-responsive sequence, which is introduced into a scaffold protein and is essential for conformation change. The Cys_2His_2-type zinc finger belongs to ubiquitous DNA binding motifs and contains the consensus sequence, "-Y/F-x-C-$x_{2,4}$-C-x_3-F-x_5-L-x_2-H-x_{3-5}-H-", where x represents relatively nonconserved amino acids. This domain folds from a disordered state into a $\beta\beta\alpha$ structure by tetrahedral Zn(II)-coordination with the conserved Cys_2His_2 residues, and the remaining conserved residues stabilize this fold, forming a hydrophobic core. These amino acid residues are advantageous for a metal-responsive sequence, because Zn(II)-binding is easily analyzed by Co(II)-substitution experiments[9] and the residues are necessary and sufficient to determine the zinc finger structure according to a previous report.[10] We anticipated that the introduced consensus residues act as a signal of Zn(II)-induced conversion of the original structure of a scaffold protein to that of the zinc finger.

The next important step is to find a scaffold protein whose structure is well-defined and is not lost by introduction of the consensus residues. The desired protein was obtained by utilizing the GenomeNet Motif (http://www.motif.genome.ad.jp). In this system, proteins with a particular sequence pattern, that is, a motif, are selected from a protein database. From PDBSTR, which is a sequence database of proteins registered on the Protein Data Bank (PDB), we searched for a protein with a motif whose inherent sequence characteristics are analogous to those of the consensus

Figure 1. Structures of Antennapedia homeodomain mutant (des(1-6)Antp-(C39S)) (PDB ID: 1SAN) (left),[8] and Sp1 finger 2 (PDB ID: 1SP2) (right),[14] typical of a C_2H_2-type zinc finger.

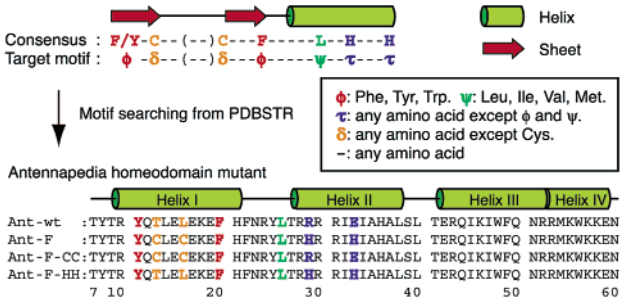

Figure 2. Design scheme of Antennafinger (Ant-F) by GenomeNet Motif.

sequence and are not altered significantly by the mutations. Considering the aromaticity, aliphaticity, hydrophilicity, and the molecular shape of side chains of the amino acids to mutate, the motif was determined to be ϕ-x-δ-$x_{2,4}$-δ-x_3-ϕ-x_5-ψ-x_2-τ-x_3-τ, where ϕ corresponding to the Tyr or Phe of the consensus sequence represents Tyr, Phe, and Trp, ψ corresponding to the Leu shows Leu, Ile, Val, and Met, τ corresponding to the His represents any amino acid except those of ϕ and ψ, δ corresponding to the Cys represents any amino acid other than Cys and precludes the zinc finger sequence in searching, and x shows any amino acid. As a result of searching for the motif, Ant-wt was selected for a scaffold among proteins found from the database. Ant-wt is a structure-determined portion of a mutant (des(1−6)Antp(C39S)) derived from Antennapedia homeodomain, which is a DNA-binding protein involved in developmental control of *Drosophila*. des(1−6)Antp-(C39S) consists of four helical regions and also binds to DNA. The reason this protein was chosen is that it is small (54 residues) and does not contain a metal ion or a disulfide bond, and its function is well understood. Therefore, Ant-F was constructed from Ant-wt with only four point mutations to 2 Cys and 2 His residues, and this protein was synthesized by the Fmoc-solid-phase technique.

The circular dichroism spectrum of apo-Ant-F displays negative Cotton effects at 208 and 222 nm, typical of an α-helical structure ($[\theta]_{222\ nm} = -19400$ deg·cm^2·dmol^{-1}), and remarkably resembles that ($[\theta]_{222\ nm} = -19600$ deg·cm^2·dmol^{-1}) of Ant-wt at 4 °C, suggesting that Ant-F has secondary structures similar to that of Ant-wt in the absence of Zn(II) (Figure 3(a)). In the presence of Zn(II), on the other hand, the negative ellipticity appreciably increases in the spectrum of Ant-F ($[\theta]_{222\ nm} =$

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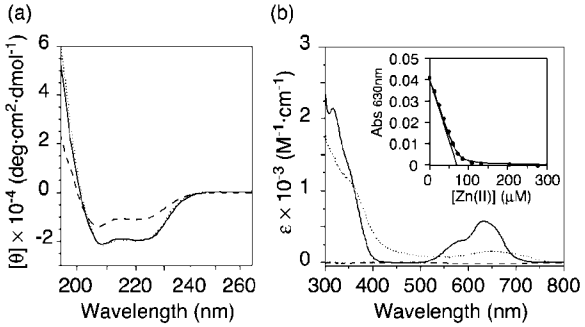

Figure 3. (a) CD spectra of Ant-wt (14.9 μM) (dotted line) and Ant-F (16.9 μM) (solid line) in the absence of Zn(II) and Ant-F (16.9 μM) (dashed line) in the presence of Zn(II) (25 μM) at 4 °C. (b) Optical absorption spectra of Co(II) complexes of Ant-F (73.3 μM) (solid line), Ant-F−CC (66.4 μM) (dotted line) and Ant-F−HH (62.5 μM) (dashed line). A saturating amount of Co(II) was added to the proteins. The spectra were corrected for the peptide and free Co(II) absorptions. The inset shows the Zn(II) titration of Ant-F (71.5 μM) in the presence of excess Co(II) (6.34 mM), monitored by the absorbance at 631 nm.

−10900 deg·cm^2·dmol^{-1}), different from the case in that of Ant-wt ($[\theta]_{222\ nm}$ = −19800 deg·cm^2·dmol^{-1}). This result clearly demonstrates that Zn(II)-binding induces a structural change in Ant-F and a decrease in the α-helical content.[11] Optical absorption spectra of the protein−Co(II) complexes were measured to investigate the metal-binding mode of Ant-F (Figure 3b). The spectrum of the complex of Co(II) and Ant-F shows absorption bands in the UV and visible regions, indicative of ligand-to-metal charge-transfer (LMCT) transitions and d−d transitions, respectively. The LMCT band indicates thiolate coordination, and the intensity ($\epsilon_{317\ nm}$ = 2130 ± 10 M^{-1}·cm^{-1}) corresponds to two Co(II)−thiolate bonds.[12] The intensity of the d−d transition band ($\epsilon_{\lambda max}$ = 539 ± 40 M^{-1}·cm^{-1}) is characteristic of a tetrahedral complex,[13] and also the band shape and position (λ_{max} = 631 nm) are similar to that of Co(II)-substituted native Cys_2His_2-type zinc fingers[14] and consistent with S_2N_2-type ligand composition.[9] Metal binding affinity was determined by optically monitored titration. Ant-F binds to Co(II) with the dissociation constant of (4.5 ± 1.7) × 10^{-5} M (see Supporting Information). In the presence of excess Co(II), the complex of Ant-F and Co(II) was titrated with Zn(II) (inset of Figure 3b). Ant-F binds to Zn(II) by replacement of Co(II) in a 1:1 stoichiometry, and the dissociation constant of (1.3 ± 0.4) × 10^{-8} M indicates the higher affinity of Zn(II) than that of Co(II) as predicted.[15] Furthermore, Ant-F−CC and −HH, each of which has only two mutations of either Cys or His residues, were synthesized to examine the involvement of the mutated Cys and His residues in the metal complex of Ant-F. In the Co(II) spectra, both of the proteins showed spectral features apparently different from that of Ant-F. This evidence strongly reveals that the mutated Cys and His residues contribute to the formation of the Co(II) complex of Ant-F.[11] Considering the coordination similarity of Zn(II) and Co-(II), these results suggest that the holo-Ant-F forms the folding structure similar to the native Cys_2His_2-type zinc finger proteins. However, a detailed structural analysis should be investigated by an NMR technique.

For a functional analysis, a gel mobility shift assay was carried out to examine whether Ant-F can regulate its DNA-binding affinity by Zn(II) (Figure 4). BS-2-28, containing the recognition sequence of Ant-wt,[8] was used in this assay (see Supporting Information). In the absence of Zn(II), Ant-F binds to BS-2-28 with the

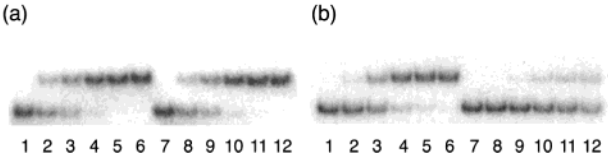

Figure 4. Gel mobility shift assay for Ant-wt (a) and Ant-F (b) bindings to BS-2-28 in the absence (lanes 1−6) or the presence (lanes 7−12) of Zn(II) (20 μM). (a) In lanes 1−6 and lanes 7−12, protein concentrations were 0, 125, 250, 500, 1000, and 2000 nM, respectively. (b) In lanes 1−6 and lanes 7−12, protein concentrations were 0, 250, 500, 1000, 2000, and 4000 nM, respectively.

dissociation constant of (6.2 ± 0.7) × 10^{-7} M. The affinity of Ant-F was 2.5-fold lower than that of Ant-wt (K_d = (2.5 ± 0.3) × 10^{-7} M)[16] but was maintained moderately. In the presence of Zn(II), by contrast, the binding affinity for BS-2-28 decreased by more than 10-fold (K_d > 8 × 10^{-6} M), whereas Ant-wt binds to BS-2-28 with almost the same affinity as in the absence of Zn(II) (K_d = (2.4 ± 0.4) × 10^{-7} M). These results show that Ant-F can regulate DNA-binding affinity through a Zn(II)-induced structural change.

We first create an artificial protein, Ant-F, which has the consensus residues of a Cys_2His_2-type zinc finger in the sequence of Ant-wt. Interestingly, Ant-F regulates its DNA binding activity through Zn(II)-triggered structural alteration. The results provide valuable information on protein dynamics and a novel concept for metalloprotein design.

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Supporting Information Available: Experimental procedures, the results of Co(II) titration to Ant-F, and CD and Co(II) spectra of Ant-F(9-35) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) The CD spectrum of Ant-F(9-35) coding the minimal region of the consensus sequence displays negative Cotton effects around 200 nm, suggesting poor secondary structure of the apo-form. In the presence of Zn(II), the negative peak shifts to 206 nm and the signal intensity of the shoulder around 218 nm increases, indicating more secondary structure of the Zn(II) complex. Similar CD features are also observed in known C_2H_2-type zinc fingers.[17] In addition, Co(II) spectrum of Ant-F(9-35) exhibits similar characteristics to those of Ant-F and native zinc fingers (see Supporting Information).[14]
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