Effects of Zn(II) Binding and Apoprotein Structural Stability on the Conformation Change of Designed Antennafinger Proteins†

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ABSTRACT: Ligand-induced conformation change is a general strategy for controlling protein function. In this work, we demonstrate the relationships between ligand binding and conformational stability using a previously designed protein, Ant-F, which undergoes a conformation change upon Zn(II) binding. To investigate the effect of stabilization of the apo structure on the conformation change, we also created a novel protein, Ant-F-H1, into which mutations are introduced to increase its stability over that of Ant-F. The chemical denaturation experiments clarified that apo-Ant-F-H1 is more stable than apo-Ant-F ($\Delta\Delta G = -1.28$ kcal/mol) and that the stability of holo-Ant-F-H1 is almost the same as that of holo-Ant-F. The Zn(II) binding assay shows that the affinity of Zn(II) for Ant-F-H1 is weaker than that for Ant-F ($\Delta\Delta G = 1.40$ kcal/mol). A large part of the increased value of free energy in stability corresponds to the decreased value of free energy in Zn(II) binding, indicating that the stability of the apo structure directly affects the conformation change. The denaturation experiments also reveal that Zn(II) destabilizes the conformation of both proteins. From the thermodynamic linkage, Zn(II) is thought to bind to the unfolded state with high affinity. These results suggest that the binding of Zn(II) to the unfolded state is an important factor in the conformational change as well as the stability of the apo and holo structures.

Creation of artificial proteins with controllable function is one of the major goals in protein chemistry. One powerful strategy for regulating protein function is a ligand-induced protein structural change (*1*−*7*). In nature, protein structural change plays an important role in various biological phenomena and is essential in modulating the protein activity to maintain constant cellular homeostasis or adapt to various conditions in a living cell (*8*−*10*). Although there are some reports about conformation changes in artificial proteins, their design strategies are limited to reengineering proteins related to a structural change upon ligand binding (*2*−*7*). On the other hand, it is still challenging to create an artificial protein, which changes its conformation on ligand binding, from a natural protein template with a single conformational state. The problem of designing such proteins is that we have little knowledge about which protein is the best as a design template or which ligand binding sequence should be introduced into the template protein for conformation change. The most valuable and indispensable approach to determining the template protein and the ligand binding sequence is obtaining information about the conformation stability and ligand binding ability of the protein. Moreover, the elucidation of their correlation provides insight into the design of proteins whose conformations can be regulated by ligand binding.

Recently, we created an artificial protein, "Antennafinger" (Ant-F),[1] inducing conformation change through Zn(II) coordination and regulating its function (*11*). Ant-F was designed by introducing the consensus sequence of C_2H_2-type zinc fingers into a natural protein, *Antennapedia* homeodomain, which does not bind Zn(II) or change its structure. A C_2H_2-type zinc finger is a structural motif of DNA binding proteins and has a highly conserved "Y/F-x-C-$x_{2,4}$-C-x_3-F-x_5-L-x_2-H-x_{3-5}-H" sequence, where x represents relatively nonconserved amino acids (*12*). The peptide consists of ∼30 amino acids and folds into a $\beta\beta\alpha$ structure by Zn(II) binding. Its structure is stabilized by Zn(II) coordination of conserved cysteines and histidines and also hydrophobic interactions among the other conserved residues. *Antennapedia* homeodomain is a DNA binding protein of *Drosophila melanogaster* and is composed of three α-helices and a short, relatively disordered helix (*13*, *14*). We previously demonstrated that Ant-F changes its structure by forming a tetrahedral metal-binding site similar to those of C_2H_2-type Zn(II) fingers and that all of the introduced Cys and His residues are involved in the coordination (*11*).

Ant-F is the most appropriate model protein for investigating the physicochemical mechanism of ligand-induced conformation change for the following reasons. First, the molecular size of Ant-F is rather small (54 amino acids), and its structure is mainly α-helical (*11*). Accordingly, Ant-F is suitable for spectroscopic analysis such as CD. Second, the Zn(II) binding is fully coupled to conformation change, and only two states are observed (*11*). Third, there are many reports about the stabilization of α-helices (*15*−*23*), and the information is helpful for stabilizing the conformation of apo-

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[1] Abbreviations: Ant-F, Antennafinger; Gdn-HCl, guanidinium hydrochloride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EGTA, *O*,*O*′-bis(2-aminoethyl)ethylene glycol-*N*,*N*,*N*′,*N*′-tetraacetic acid; CD, circular dichroism; ASA, solvent-accessible surface area.

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Ant-F, which is composed of α-helical structures. Examining the influence of stabilization of an apo structure on the conformation change can provide clues for understanding the thermodynamic driving force for ligand-induced conformation change. In this study, metal binding and chemical unfolding experiments were carried out to elucidate the relationships between Zn(II) binding and the conformation stability of Ant-F on protein conformation change. In addition, the effect of stabilization of Ant-F on Zn(II) binding was also investigated.

EXPERIMENTAL PROCEDURES

Peptide Syntheses. The syntheses of Ant-F and Ant-F-H1 were conducted by the Fmoc solid-phase technique on a Rink amide resin. The protein chain (residues 17−60) was automatically constructed with a Shimadzu PSSM-8 synthesizer, using its standard protocol with the benzotriazol-1-yloxytrispyrrolidinophosphonium hexafluorophosphate (PyBOP)/1-hydroxybenzotriazole (HOBt)/*N*-methylmorpholine (NMM) coupling system. The remaining chain (residues 7−16) was constructed by the same method, although a longer reaction time was required for the coupling. The protected protein resin was treated with a trifluoroacetic acid/ethanedithiol mixture (95:5) at room temperature for 2 h, followed by HPLC purification on a COSMOSIL $5C_{18}$-AR-II (10 mm × 250 mm) column. The fidelity of the products was confirmed by time-of-flight mass spectrometry (TOFMS), using a Voyager-DE STR system (Applied Biosystems): Ant-F [MH^+] calcd 7009.2, observed 7010.2; Ant-F-H1 [MH^+] calcd 7053.2, observed 7054.3. The purified proteins were lyophilized and dissolved in buffers just before experiments were conducted. Buffers were preserved in low-density polyethylene bottles washed with 4 N HCl to remove trace metal ions from the bottles. The contamination of trace metal ions in buffers can be neglected in the peptide concentration (>10 μM) of this study.

CD Measurements. The CD spectra of Ant-F and Ant-F-H1 were recorded on a Jasco J-720 spectropolarimeter in Tris-HCl buffer (pH 7.5) containing 50 mM NaCl at 15 °C in a 0.1 cm path length cell under a nitrogen atmosphere.

Evaluation of Chemical Denaturation. CD data were obtained on the spectropolarimeter equipped with a PTC-343 temperature controller. Chemical denaturation with Gdn-HCl was conducted in Tris-HCl buffer (pH 7.5) containing 50 mM NaCl at 15 °C in a 0.2 cm path length cuvette under a nitrogen atmosphere and monitored by CD ellipticity at 222 nm. Data were acquired with an averaging time of 60 s after equilibration for 5 min. The free energy of unfolding was determined from the chemical denaturation data assuming a two-state transition and using the linear extrapolation model (*24*):

$$\Delta G_u(D) = \Delta G_u(H_2O) + m[D]$$

where $\Delta G_u(D)$ represents the free energy of unfolding in the presence of a denaturant, $\Delta G_u(H_2O)$ the free energy in the absence of a denaturant, and m a measure of the dependence of $\Delta G_u(D)$ on denaturant concentration.

Metal Binding Study. Zn(II) titration experiments were conducted in Tris-HCl buffer (pH 7.5) containing 50 mM NaCl and 1 mM EGTA at 15 °C in a 0.2 cm path length cuvette under a nitrogen atmosphere and monitored by CD ellipticity at 222 nm. On the basis of the CD signal changes, the Zn(II)-bound fraction, Φ, was determined by

$$\Phi = ([\theta]_i - [\theta]_{int})/([\theta]_{fin} - [\theta]_{int})$$

where $[\theta]_i$ is the CD ellipticity measured after the ith addition of Zn(II) and $[\theta]_{int}$ and $[\theta]_{fin}$ represent the CD ellipticities of the apo and fully Zn(II)-saturated proteins, respectively. At each Φ, the concentration of free Zn(II), $[Zn(II)]_f$, is estimated from the following quadratic equation (*25*, *26*):

$$K_E[Zn(II)]_f^2 + [K_E([E]_t - [Zn(II)]_t + \Phi[P]_t) + 1] \times [Zn(II)]_f + \Phi[P]_t - [Zn(II)]_t = 0$$

where $[E]_t$, $[Zn(II)]_t$, and $[P]_t$ are the total concentration of EGTA, Zn(II), and protein, respectively, while K_E is the association constant for association of EGTA and Zn(II) under these conditions ($K_E = 1.01 \times 10^9\ M^{-1}$) (*27*, *28*). The association constants of the proteins and Zn(II) were determined by fitting the obtained values of Φ and $[Zn]_f$ to the following equation (*25*, *26*):

$$\Phi = K_a[Zn]/(1 + K_a[Zn]_f)$$

Electronic Absorption Spectroscopy. Electronic absorption spectra were recorded on a Beckmann DU640 spectrophotometer in Tris buffer (pH 7.5) containing 50 mM NaCl under a nitrogen atmosphere.

RESULTS

Designs and Creations of Ant-F and Ant-F-H1. To investigate the effect of the stabilization of apo-Ant-F on Zn(II) binding and conformation change, Ant-F-H1 was created. Previous reports showed that apo-Ant-F mainly consists of a helical structure similar to its wild-type protein (*11*). According to statistical investigations of natural proteins (*15*, *16*) and studies on model peptides (*17*−*19*), the stability of α-helices significantly depends on the helix propensity of the amino acids of which the helix consists, the helix dipole, and N-capping effects. Amino acids such as Ala, Leu, Glu, Gln, Lys, and Met have a strong tendency to form a helix. Natural proteins rich in helices or model peptides of helices often contain this type of amino acid to stabilize an α-helix. A helix dipole, which has a partial positive charge at the N-terminus and a partial negative charge at the C-terminus, is generated due to the lack of backbone hydrogen bonds of four amides at both the N-terminus and C-terminus of the helix (*20*, *21*). Preferable electrostatic interactions of the amino acids with the helix dipole at the N- or C-terminus of the helix have been demonstrated to increase the stability of the helix. N-Capping effects refer to the hydrogen bonding interaction between an N-cap residue, which immediately precedes the N-terminal side of the helix, and the backbone amide which is located at the N-termini and is not involved in hydrogen bonding with any other backbone carbonyl. The side chain of Ser, Thr, Asn, or Asp has been shown to interact favorably with the backbone (*20*−*23*). (Indeed, all the N-cap residues of the wild-type *Antennapedia* homeodomain are composed of Thr.)

Considering the stabilization mechanism of helices described above, we designed Ant-F-H1 to stabilize the conformation of the helix in Ant-F (Figure 1). The mutations

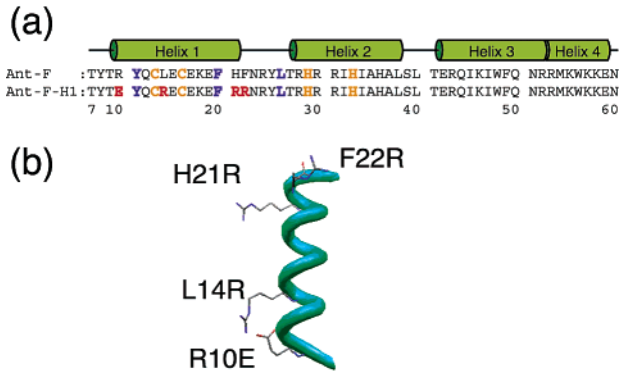

FIGURE 1: (a) Primary sequences of Ant-F and Ant-F-H1. The mutated amino acids are shown in red. The residues probably involved in Zn(II) coordination are shown in orange. (b) Model structure of helix 1 in Ant-F-H1. The model was generated from the PDB structure of *Antennapedia* homeodomain (PDB entry 1SAN) utilizing Swiss-PdbViewer version 3.0. The mutated amino acids are displayed.

were introduced into helix 1, because helix 1 lies within the consensus sequence of the zinc finger motif and is thought to participate in metal binding and conformation change. First, Arg10 was replaced with Glu, which has a strong tendency to form an α-helix and interacts with the partial positive charge of the helix dipole. Next, both His21 and Phe22 were substituted with Arg to neutralize the negative charge of the helix dipole. Arg was selected in these positions, because previous reports showed that these mutations increased the conformational stability of homeodomain fold (*29*). In addition, Leu14 was changed to Arg, because the hydrophobic residue is considered to have destabilizing effects in this position which is exposed to the solvent. The amino acid with a positive charge is chosen to avoid electrostatic repulsion with the adjacent Glu and has a favorable ($i-i+4$) interaction with the mutated Glu at the N-terminus. All the positions of the mutation are selected at surface positions so the packing interactions inside the protein would not be disturbed.

Denaturations and Stabilities of Ant-F and Ant-F-H1. Figure 2 shows the CD spectra of Ant-F and Ant-F-H1 in the absence or presence of 4 M Gdn-HCl. Addition of Zn(II) to apo-Ant-F induces a structural change (a less α-helical conformation). The CD spectra in the presence of 4 M Gdn-HCl indicate that both apo- and holo-Ant-F lose their α-helical structures and are denatured by 4 M Gdn-HCl. The CD features of Ant-F-H1 show that Ant-F-H1 has secondary structures similar to those of Ant-F in the absence of Zn(II) and also binds Zn(II), leading to a decrease in α-helicity. The addition of 4 M Gdn-HCl destroyed the ordered secondary structures of both apo- and holo-Ant-F-H1 as well as Ant-F. To analyze the conformation stability in detail, isothermal denaturation experiments were conducted with Gdn-HCl titration (Figure 3). The chemical denaturation data were fit to a two-state model. The transition midpoints (C_m) at which 50% of the proteins are unfolded, the slope of the plot of ΔG_u(D) versus Gdn-HCl concentration, m, and the free energy of unfolding in the absence of denaturant, ΔG_u-(H_2O), are summarized in Table 1. The values of C_m decreased by 0.17 M for Ant-F and 0.82 M for Ant-F-H1, and the $-m$ values also decreased by 0.57 kcal mol^{-1} M^{-1} for Ant-F and 0.81 kcal mol^{-1} M^{-1} for Ant-F-H1 as a result

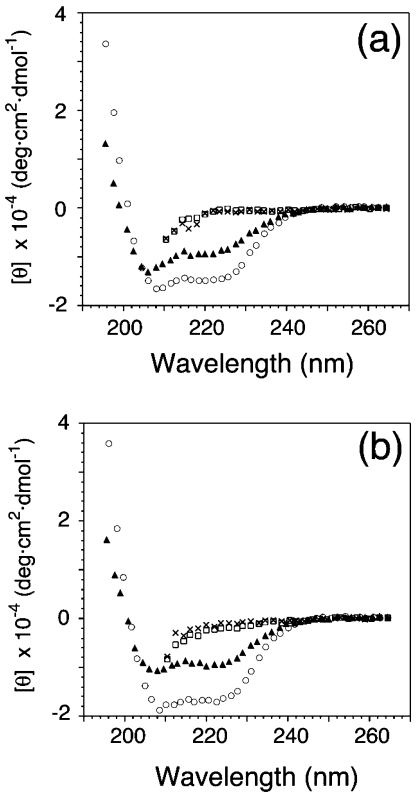

FIGURE 2: CD spectra of (a) Ant-F (13.5 μM) and (b) Ant-F-H1 (13.8 μM). The circles and triangles show the spectra of the apo- and holoproteins in the absence of Gdn-HCl, respectively. The squares and crosses represent the spectra of the apo- and holoproteins in the presence of 4 M Gdn-HCl, respectively. The holoproteins were prepared by addition of $ZnCl_2$ (20.0 μM).

of addition of Zn(II). The unfolding free energy of each holoprotein was lower than that of the corresponding apoprotein, indicating that Zn(II) binding has destabilizing effects on both proteins. The $\Delta G_u(H_2O)$ value of apo-Ant-F-H1 was 1.28 kcal/mol higher than that of apo-Ant-F. This result demonstrates that the mutations introduced into Ant-F increase the stability of the apo conformation as expected. On the other hand, the $\Delta G_u(H_2O)$ values of Ant-F and Ant-F-H1 were almost the same in the presence of Zn(II), suggesting that the mutations have little effect on the stability of the holo structure.

Zn(II) Binding Constants of Ant-F and Ant-F-H1. Zn(II) binding constants were determined by direct titration of Zn(II) into a protein solution containing 100 μM EGTA (Figure 4). The data were obtained from the change in the CD signal at 222 nm, which indicates a conformation change induced by Zn(II) binding. Therefore, Zn(II) binding was monitored indirectly through the conformation change. The binding constants of Ant-F and Ant-F-H1 were determined to be (3.89 ± 0.32) × 10^9 and (3.33 ± 0.21) × 10^8 M^{-1}. The Zn(II) binding constant of Ant-F is higher than the previously determined value (*11*). This result is probably due to different experimental conditions and assay methods. From these parameters, the free energies of Zn(II) binding at 288 K were calculated to be −12.6 and −11.2 kcal/mol for Ant-F and Ant-F-H1, respectively. The errors in the calculated values obtained from three independent experiments were negligible. Therefore, these results indicate that Ant-F binds Zn(II) better than Ant-F-H1 does.

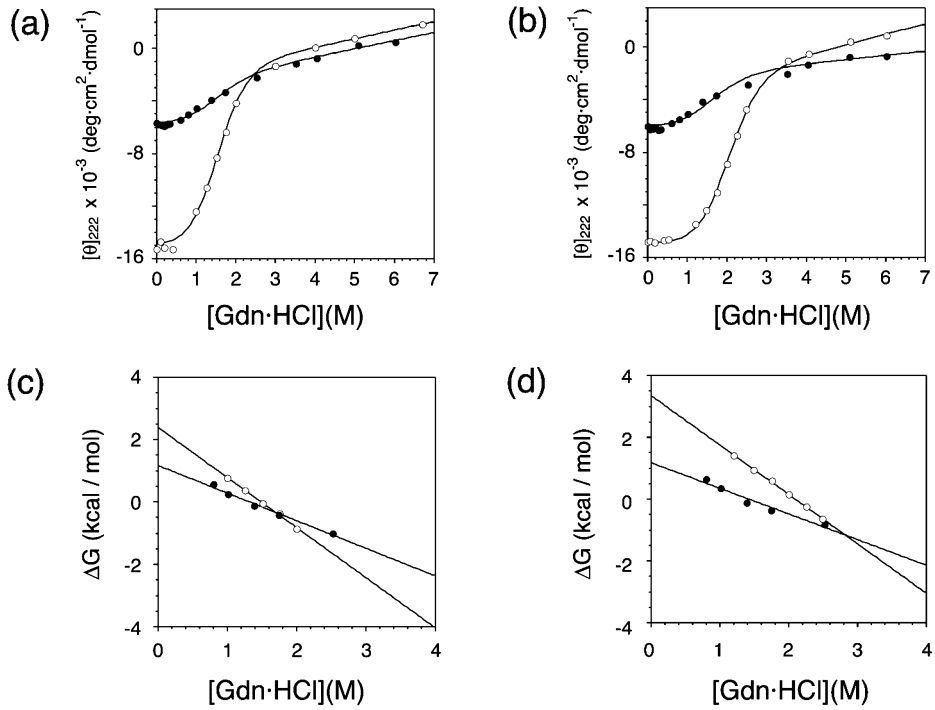

FIGURE 3: Gdn-HCl denaturation of (a) Ant-F and (b) Ant-F-H1 at 288 K. The unfolding was monitored with $[\theta]_{222}$. The data in the transition region of denaturation are converted to ΔG values and plotted as a function of denaturant concentrations in panels c (Ant-F) and d (Ant-F-H1). The empty and filled circles represent the denaturation of the apo- and holoproteins, respectively, in each graph. The peptide concentration ranges from 10 to 12 μM. The complexes of proteins and $ZnCl_2$ were formed by adding 3 molar equiv of $ZnCl_2$ to the proteins.

Table 1: Summary of Conformational Stability and Zn(II) Binding Affinity

	C_m (M)	m (kcal mol^{-1} M^{-1})	ΔG_u (kcal/mol)	K_a (M^{-1})	$\Delta G_{Zn(II)}$ (kcal/mol)
apo-Ant-F	1.49 ± 0.02	−1.44 ± 0.08	2.14 ± 0.10	(3.89 ± 0.32) × 10^9	−12.6
holo-Ant-F	1.32 ± 0.06	−0.88 ± 0.08	1.16 ± 0.16		
apo-Ant-F-H1	2.14 ± 0.04	−1.60 ± 0.06	3.43 ± 0.12	(3.33 ± 0.21) × 10^8	−11.2
holo-Ant-F-H1	1.32 ± 0.20	−0.79 ± 0.08	1.04 ± 0.13		

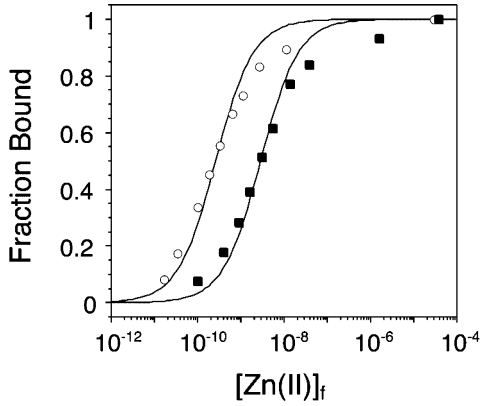

FIGURE 4: Zn(II) binding assays with Ant-F (○) and Ant-F-H1 (■) in the presence of 100 μM EGTA. Each peptide concentration is 10 μM. The data of the protein fractions bound to Zn(II) were obtained by monitoring the decrease in CD negative Cotton effects at 222 nm due to Zn(II)-induced reduction in the level of α-helical structures and fitted to the theoretical curves, as described in Experimental Procedures.

Metal Binding under Native and Denaturation Conditions. Co(II) absorption spectra of the designed proteins under native and denaturation conditions were recorded to confirm metal binding to the unfolded proteins as well as the folded proteins. The above-mentioned CD experiments show that 4 M Gdn-HCl destroys the secondary structures of the apo- and holoproteins. Therefore, the proteins under denaturation conditions were prepared by dissolving the proteins in the buffer containing 4 M Gdn-HCl. Co(II) is often used as a spectroscopic probe of Zn(II) because Zn(II) itself displays no characteristic absorption band (*30*, *31*). The spectra in the absence of Gdn-HCl exibit the visible and UV absorption bands assigned to d−d transitions and ligand-to-metal charge transfers (LMCT), respectively (Figure 5a,b) (*30*−*32*). The LMCT bands indicate that both the proteins bind to thiolate of the Cys residues (*30*, *31*). The amplitude, shape, and position of the d−d transition bands reveal that each of the proteins forms a S_2N_2-type tetrahedral complex with two Cys and two His residues as possible ligands under native conditions (*30*−*32*). Surprisingly, the Co(II) spectra in the presence of 4 M Gdn-HCl clearly exhibit spectra similar to those in the absence of Gdn-HCl (Figure 5c,d). In the spectra of Ant-F and Ant-F-H1, absorption bands indicative of d−d transitions and LMCT were also observed in the visible and UV regions, respectively. The spectral features resemble those under native conditions and those of Co(II)-replaced natural zinc finger peptides, suggesting that the proteins presumably form S_2N_2-type tetrahedral complexes with two Cys and two His residues even under denaturation conditions. Upon addition of Zn(II) to the Co(II)-bound proteins, the absorption of the Co(II) complexes of the proteins vanished under both native and denaturation conditions (Figure 5).

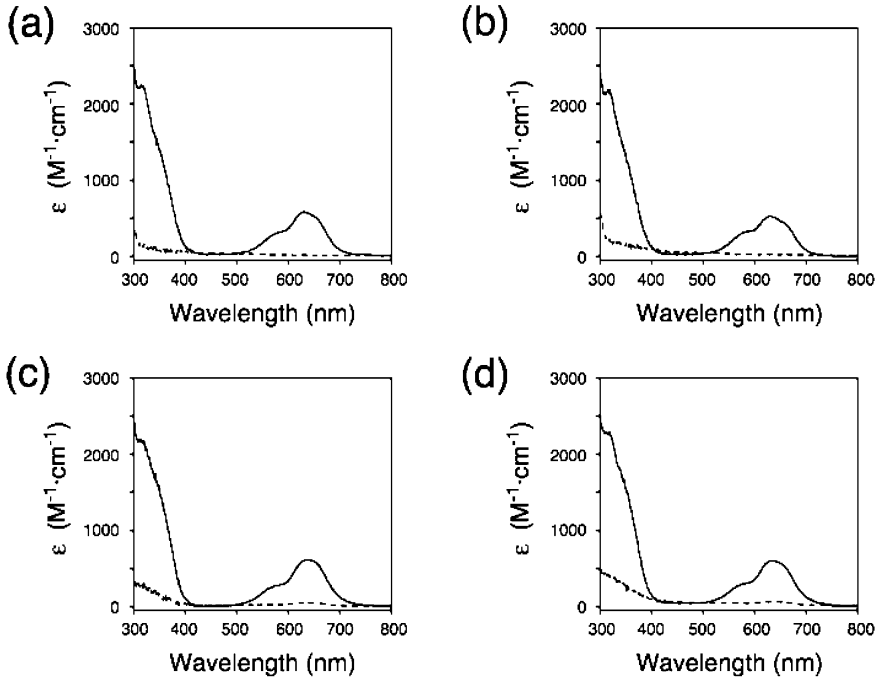

FIGURE 5: Electronic absorption spectra of Co(II) and Zn(II) complexes of Ant-F (a and c) and Ant-F-H1 (b and d). Panels a and b show the spectra of the complexes in the absence of a denaturant and panels c and d in the presence of 4 M Gdn-HCl. The solid lines represent the spectra of the proteins (30 μM) in the presence of Co(II) (90 μM). The dashed lines represent the spectra of the proteins (30 μM) to which Zn(II) (45 μM) was added after the addition of Co(II) (90 μM).

These results demonstrate that Zn(II) binds to the native and Gdn-HCl-denatured proteins by replacing the Co(II) bound to the proteins.

DISCUSSION

From the denaturation experiments, the $-m$ values were found to be lower in both Ant-F and Ant-F-H1 due to Zn(II) binding. The value of $-m$ is proportional to the change in the solvent-accessible surface area (ASA) on unfolding (*33*−*35*). One plausible reason for the decrease in the $-m$ value is that the protein structure became more extended with the Zn(II)-induced conformation change, leading to the increase in the ASA of the folded holo conformation. Another reason is the decrease in the ASA of the unfolded conformation in the presence of Zn(II). The $-m$ values of proteins with disulfide bonds or other cross-links are known to decrease because the cross-links in the unfolded state form a more compact conformation, leading to a reduction of the ASA in the unfolded state (*33*). Therefore, the decrease in the $-m$ value suggests that Zn(II) binds to the unfolded state and that the Zn(II)-linked unfolded structure is more compact than that of the unfolded apo form.

The stability of both Ant-F and Ant-F-H1 decreased due to Zn(II) binding. On the other hand, the metal binding assays showed that Zn(II) binds to both the proteins strongly with nanomolar dissociation constants. To explain that the structures of designed proteins change due to Zn(II) binding despite the destabilizing effects of Zn(II) on the conformational stability, a possible thermodynamic model of a Zn(II)-induced conformation change is proposed in Figure 6a. In this model, the unfolded states of the proteins are able to bind to Zn(II) as well as the folded state. The values of the Zn(II) binding energy are calculated on the basis of the model

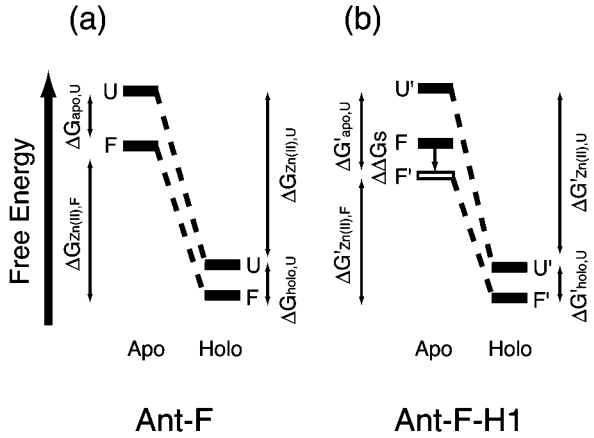

FIGURE 6: Estimated models of the relationships between protein stability and Zn(II) binding upon the conformation change in (a) Ant-F and (b) Ant-F-H1. F and U represent the energy levels of the folded and unfolded states, respectively, in Ant-F. F′ and U′ also show the energy levels of the folded and unfolded states, respectively, in Ant-F-H1. $\Delta\Delta G$ designates the difference in the unfolding free energies between apo-Ant-F and Ant-F-H1.

as follows:

$$\Delta G_{\mathrm{Zn(II),U}} = -\Delta G_{\mathrm{apo,U}} + \Delta G_{\mathrm{Zn(II),F}} + \Delta G_{\mathrm{holo,U}}$$

where $\Delta G_{\mathrm{Zn(II),U}}$, $\Delta G_{\mathrm{apo,U}}$, $\Delta G_{\mathrm{Zn(II),F}}$, and $\Delta G_{\mathrm{holo,U}}$ represent the free energies of Zn(II) binding in the unfolded state, unfolding in the absence of Zn(II), Zn(II) binding in the folded state, and unfolding in the presence of Zn(II), respectively.

The values of $\Delta G_{\mathrm{Zn(II),U}}$ were estimated to be −13.6 kcal/mol for both the proteins, indicating that the unfolded states bind to Zn(II) with fairly high affinity. Although a natural zinc finger protein does not have any particular conformation

in the absence of Zn(II), the unfolded state of the zinc finger binds to Zn(II) and simultaneously folds into the native structure. The thermodynamic analysis suggests that the unfolded states of both the proteins also have Zn(II) binding ability like the natural zinc finger protein. Indeed, the present proteins are designed to have the consensus residues of the zinc finger.

The event during which Zn(II) binds to the unfolded states is supported by the decrease in the $-m$ values and the results of the spectroscopic experiments under denaturation conditions. The CD experiments show that the proteins lose their secondary structures in the presence of 4 M Gdn-HCl. However, the absorption spectra indicate the denatured proteins still bind to Co(II) by forming tetrahedral complexes and also bind to Zn(II). Interestingly, judging from the spectral shape and position, two Cys and two His residues are presumably ligands to metal ion even in the denatured holoproteins as well as in the native holoproteins. Although the conformation in the presence of 4 M Gdn-HCl might be different from the denatured conformation in the absence of Gdn-HCl, these results strongly suggest that metal ion binds to the unfolded state of Ant-F and Ant-F-H1 in the absence of a denaturant (under native conditions).

The results for chemical denaturation of apo-Ant-F-H1 demonstrate that the mutations introduced into helix 1 of Ant-F stabilize the conformation of apo-Ant-F-H1 by 1.28 kcal/mol. On the other hand, Zn(II) titration experiments show that the Zn(II) binding affinity of Ant-F-H1 is approximately 10-fold lower than that of Ant-F. The decreased value in binding energy almost corresponds to the increased value in the conformational stability. The model illustrated in Figure 6b indicates that the stability of the apo structure affects the binding energy of Zn(II).

As Zn(II) binding in the folded state is completely coupled to the conformation change, the binding energy for binding of Zn(II) to the folded conformation can be regarded as an indicator of the conformation change. The models suggest that the determinant of conformation change is the difference between the free energies of the folded apo and holo structures. The alteration of the stability in the apo form affects the conformation change. However, the change in the stability of the holo form is not necessarily correlated with the conformation change, because Zn(II) is thought to bind to the unfolded state and the Zn(II) binding to the unfolded state is one of the factors influencing the free energy balance between the folded apo and holo conformations as well as the stability of the folded holo conformation. Metal binding to the unfolded states of natural or engineered proteins, which are not related to conformation change, has been reported (*36*−*38*). The authors suggest that one possible factor in a metal binding to the unfolded states is the proximity among the ligand amino acids in the primary sequence. In the case of Ant-F and Ant-F-H1, the spectroscopic experiments with the Co(II) complexes suggest that probable ligands of the metal ion under denaturation conditions are Cys13, Cys16, His29, His33, and His36 (His21 is also possible in Ant-F). The number of intervening residues between these amino acids is relatively small (at most 19−22 residues). Although the ligands of the unfolded proteins in the absence of a denaturant are not identified, the proximity among metal binding residues in the unfolded states might contribute to the binding of Zn(II) to the unfolded states, and hence play an important role in the conformation change.

In conclusion, there are three important factors in the protein conformation change induced by Zn(II), namely, the stability of the folded apo structure, the stability of the folded holo structure, and the binding of Zn(II) to the unfolded state. One possible factor in the binding is the proximity among metal binding residues. Binding of a ligand to the unfolded state is one of the important concepts in the design of proteins which control their structures by ligand binding.

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