BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

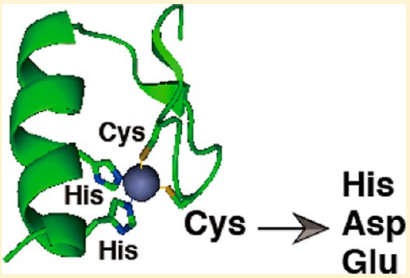
Zn(II) Binding and DNA Binding Properties of Ligand-Substituted CXHH-Type Zinc Finger Proteins

Miki Imanishi,*[,†] Kazushi Matsumura,[†] Shogo Tsuji,[†] Tomohiro Nakaya,[†] Shigeru Negi,[‡] Shiroh Futaki,[†] and Yukio Sugiura[‡]

[†]Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan

[‡]Faculty of Pharmaceutical Sciences, Doshisha Women's University, Kyotanabe, Kyoto 610-0395, Japan

ABSTRACT: CCHH-type zinc fingers are among the most common DNA binding motifs found in eukaryotes. In a previous report, we substituted the second ligand cysteine residue with aspartic acid, producing a Zn(II)-responsive transcription factor; this indicates that a ligand substitution is a possible design target of an engineered zinc finger peptide. Despite the importance of Zn(II) binding with respect to the folding and DNA binding properties of a zinc finger peptide, no study about the effects of ligand substitution on both Zn(II) binding and DNA binding properties has been reported. Here, we substituted a conserved cysteine (C) with other zinc-coordinated amino acid residues, histidine (H), aspartic acid (D), and glutamic acid (E), to create CXHH-type zinc finger peptides (X = C, H, D, and E). The Zn(II)-dependent conformational change was observed in all peptides; however, the Zn(II) binding affinity and metal coordination geometry of the peptides were different. Gel mobility shift assays showed that the Zn(II)-bound forms of the ligand-substituted derivatives retain DNA binding ability, while the DNA binding affinity decreased in the following manner: CCHH > CDHH > CEHH ≫ CHHH. The DNA binding sequence preferences of the ligand-substituted derivatives were similar to that of the wild type in the context of the full three-finger DNA-binding domain of transcription factor Zif268. These results indicate that artificial zinc finger proteins with various DNA binding affinities that respond to a diverse range of Zn(II) concentrations can be designed by substituting the Zn(II) ligand.



The classical CCHH-type zinc finger motif is among the most common DNA binding motifs found in eukaryotes. The motif has been used as a template to engineer artificial DNA-binding proteins because of its characteristic DNA recognition ability. The motif contains a highly conserved sequence, (Phe/Tyr)-X-Cys-X_{2-4}-Cys-X_3-(Phe/Tyr)-X_5-Leu-X_2-His-X_{3-5}-His, where X represents any amino acid.[1,2] In this motif, Zn(II)-binding sites serve a structural role. Binding of Zn(II) to the peptide through conserved cysteine and histidine residues is necessary for proper folding into a globular $\beta\beta\alpha$ structure and for DNA binding.[3] In typical CCHH zinc finger peptides such as Zif268 zinc fingers, Zn(II) binding is quite stable; thus, under physiological conditions, the peptides can always bind DNA. We previously created a CDHH zinc finger peptide by substituting the second ligand cysteine residue of the second Zif268 zinc finger with aspartic acid.[4] The CDHH zinc finger showed an affinity for Zn(II) 500-fold lower than that of the wild-type (WT) peptide (CCHH), resulting in Zn(II)-responsive DNA binding. This allowed us to manipulate the DNA binding affinity of the CDHH-type zinc finger protein by changing the zinc ion concentration. These results suggest that ligand substitution is a potential design target of engineered DNA-binding zinc finger peptides. To date, however, efforts to engineer zinc finger peptides have focused on mutations of the DNA recognition helix.[5−10]

To further examine the effects of ligand substitution on metal and DNA binding, we substituted the second cysteine with other amino acids that may coordinate Zn(II). With few exceptions, the donors for Zn(II) coordination found in natural proteins are sulfur from cysteine (C), imidazole nitrogens from histidine (H), and carboxylate oxygen(s) from aspartic acid (D) or glutamic acid (E).[11,12] Relatively few studies have reported ligand substitutions in CCHH-type zinc finger proteins.[13−17] Mackay et al. examined the Zn(II)-dependent conformational changes and DNA binding properties of several CCHX zinc finger derivatives.[16] However, no other systematic study combining the Zn(II) binding and DNA binding properties of ligand-substituted zinc fingers has been reported. Investigations of the structural properties and DNA binding ability of ligand-substituted zinc finger peptides should provide useful information for the design of artificial DNA-binding proteins.

In this study, we created CHHH- and CEHH-type zinc finger peptides, in addition to the previously reported CCHH-type (WT) and CDHH-type peptides. The Zn(II) binding and folding properties of these ligand-substituted peptides were spectroscopically examined. We also prepared three-zinc finger peptides, ZF3(CXHH) (X = C, H, D, and E), in which the second finger of the Zif268 zinc finger domain was substituted with the CXHH-type to investigate the DNA binding characteristics.

Received: February 21, 2012
Revised: April 5, 2012
Published: April 6, 2012

   dx.doi.org/10.1021/bi300236m | *Biochemistry* 2012, 51, 3342−3348

MATERIALS AND METHODS

Peptide Synthesis and Purification. The synthesis of f2(CXHH) was conducted by Fmoc-solid-phase synthesis on a Rink amide resin, such that each peptide has a C-terminal amide structure.[4] The fidelity of the products was confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) using a microflex system (Bruker Daltonics): f2(CHHH) [M + H^+] calcd 3441.7, observed 3441.9; f2(CEHH) [M + H^+] calcd 3433.5, observed 3433.8. As for f2(CCHH) and f2(CDHH), the previously synthesized peptides were used.[4]

UV−Vis Absorption Spectroscopy. The UV−vis absorption spectra of f2(CXHH) peptides (100 μM) were recorded on a Beckman Coulter DU640 spectrophotometer at room temperature in 10 mM Tris-HCl (pH 7.5) buffer containing NaCl (100 mM) and 250 μM $CoCl_2$ in a 1 cm path-length cell. Subsequently, 500 μM $ZnSO_4$ was added to the samples. All the presented spectra were normalized using the equation $\varepsilon = A/lc$, where ε is the extinction coefficient (M^{-1} cm^{-1}), A is the absorbance, l is the path length of the cell (centimeters), and c is the peptide concentration (molar).

Circular Dichroism Spectroscopy. The CD spectra for f2(CXHH) were recorded on a Jasco J-820 spectropolarimeter in 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 60 μM zinc finger peptide in the presence and absence of 20, 40, 60, and 80 μM $ZnSO_4$ at 20 °C in a 0.1 cm path-length cell under an atmosphere of nitrogen. The spectra were recorded from 200 to 260 nm in continuous mode with a 1 nm bandwidth, a 1 s response, and a scan speed of 50 nm/min. The Zn(II) titration experiments were conducted by monitoring the CD ellipticity at 222 nm under the competitive conditions between zinc finger peptides and EGTA, as described previously.[4] Variable-temperature experiments were performed using 30 μM zinc finger peptide in the presence of 100 μM $ZnSO_4$ at 2 °C increments between 20 and 80 °C with an equilibration time of 2 min.

Construction of Plasmids and Preparation of Proteins. The expression vectors for ZF3(CXHH) were constructed as previously described,[4] based on ZifZF3-pEV3b.[18] ZF3(CXHH) proteins were expressed in *Escherichia coli* strain BL21(DE3) (Novagen) and purified as previously described.[4]

Electrophoretic Mobility Shift Assays (EMSAs). The EMSAs were conducted under the following conditions. Each reaction mixture contained 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM dithiothreitol, 10 μM $ZnSO_4$, 0.05% Nonidet P-40, 20 ng/μL calf thymus DNA, 40 ng/μL BSA, 5% glycerol, 2.5 nM FITC end-labeled target DNA fragment, the serially diluted zinc finger protein, and/or 100 μM EDTA. After being incubated at 20 °C for 3 h, the samples were electrophoresed in an 8% polyacrylamide gel with 89 mM Tris-borate buffer at room temperature. The bands were visualized using an LAS9000 detection system (GE Healthcare) and analyzed using ImageJ. The equilibrium dissociation constant (K_d) of each protein−DNA fragment complex was evaluated by fitting the experimentally obtained values for the fraction of labeled DNA bound to the protein (θ_b) to eq 1 using KaleidaGraph (Abelbeck software).

$$\theta_b = \left[[P] + [D] + K_d - \sqrt{([P] + [D] + K_d)^2 - 4[P][D]} \right] / (2[D]) \quad (1)$$

In the EMSA for examining DNA binding selectivity, 64 FITC end-labeled DNAs containing a Zif268 targeting sequence (5′-GCG NNN GCG-3′) were used. The 64 template oligonucleotide DNAs [5′-GATCG AATTC GGAAA ACGCn nnCGC TTCAA GGATC CGTCT-3′ (n = A, C, G, or T)] were polymerized using a 5′-FITC-labeled primer (5′-AGACG GATCC TTGAAG-3′). The EMSAs were performed in the same buffer described above with a constant concentration of each zinc finger peptide, at which the ratio of the shift band for NNN = TGG becomes ~90%.

RESULTS AND DISCUSSION

Association of Zn(II) with the Ligand-Substituted Zinc Finger Mutants. We synthesized and evaluated CXHH-type single-finger peptides, f2(CXHH) (X = C, H, D, and E) (Figure 1A). The metal−peptide coordination chemistry was investigated by UV−vis absorption spectroscopy. Because Zn(II) is a spectroscopically silent ion in the visible region of the electromagnetic spectrum, Co(II) was used as a spectroscopic probe for the zinc site.[3] The UV−vis spectra of the Co(II) complexes of the f2(CXHH) peptides are shown in Figure 2. The intense absorption bands in the near-UV regions are indicative of the Cys-S^-−Co(II) ligand-to-metal charge transfer (LMCT) transition.[19] The magnitude of the extinction coefficient (ε) at 320 nm reflects the number of thiolate groups coordinated to the metals and averages ~900−1200 M^{-1} cm^{-1} per S^-−Co(II) bond.[20,21] It is also known that optical transitions of a tetrahedral Co(II) species exhibit intense d−d absorption bands (ε > 300 M^{-1} cm^{-1}) in the region of 625 ± 50 nm, derived from a relatively small ligand field stabilization energy.[22] In this study, the ε values at 320 nm were 1840 M^{-1} cm^{-1} for f2(CCHH) and 950 M^{-1} cm^{-1} for f2(CHHH), suggesting that f2(CCHH) and f2(CHHH) use two thiol groups and one thiol group to coordinate with Co(II), respectively. Intense absorption bands around 625 nm were also observed for f2(CCHH) and f2(CHHH). These

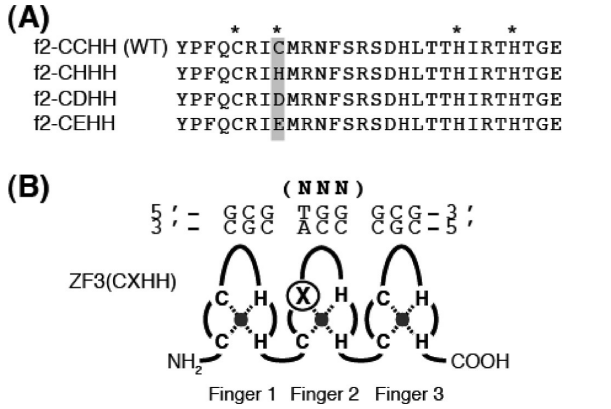


Figure 1. (A) Amino acid sequences of ligand-substituted finger 2 peptides of the Zif268 zinc finger domain. The second cysteine residue was substituted with histidine, aspartic acid, or glutamic acid. Asterisks denote the conserved Zn(II) ligands of the CCHH-type zinc finger domains. The positions of ligand substitution are shaded. (B) Schematic representation of a three-finger protein, ZF3(CXHH), containing a CXHH-type finger at finger 2 of the three-zinc finger domain of Zif268. The DNA sequence indicates the typical Zif268 binding sequence. The DNA triplet recognized by finger 2 was randomized to NNN (N = A, C, G, or T) for the DNA binding specificity experiments.

dx.doi.org/10.1021/bi300236m | *Biochemistry* 2012, 51, 3342−3348

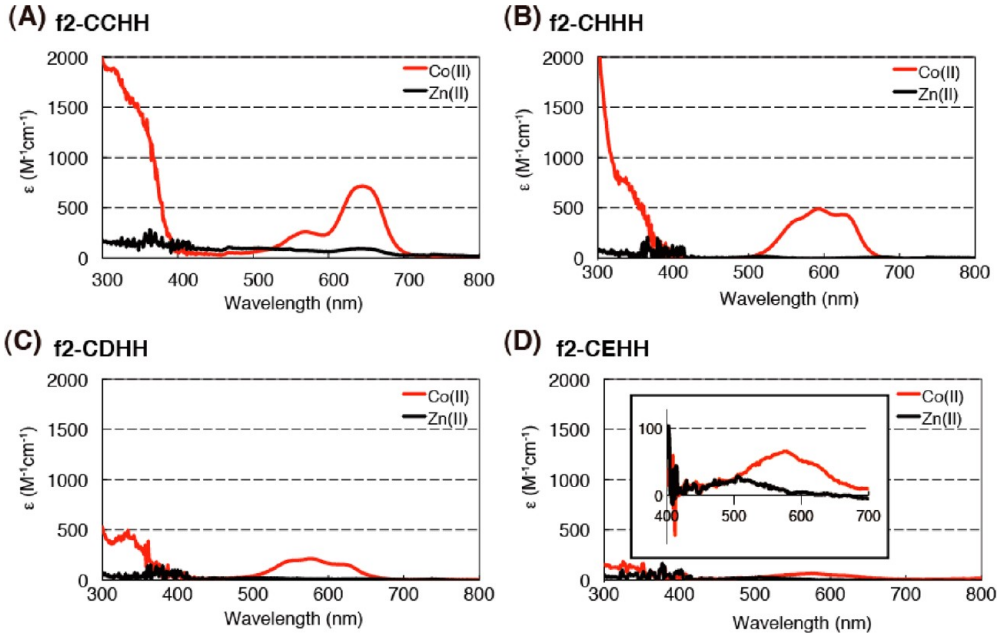


Figure 2. UV−vis absorption spectra of f2(CXHH) peptides (100 μM) in the presence of 250 μM Co(II) (red) and following the addition of 500 μM Zn(II) (black): (A) f2(CCHH), (B) f2(CHHH), (C) f2(CDHH), and (D) f2(CEHH). The inset in panel D represents enlarged spectra between 400 and 700 nm.

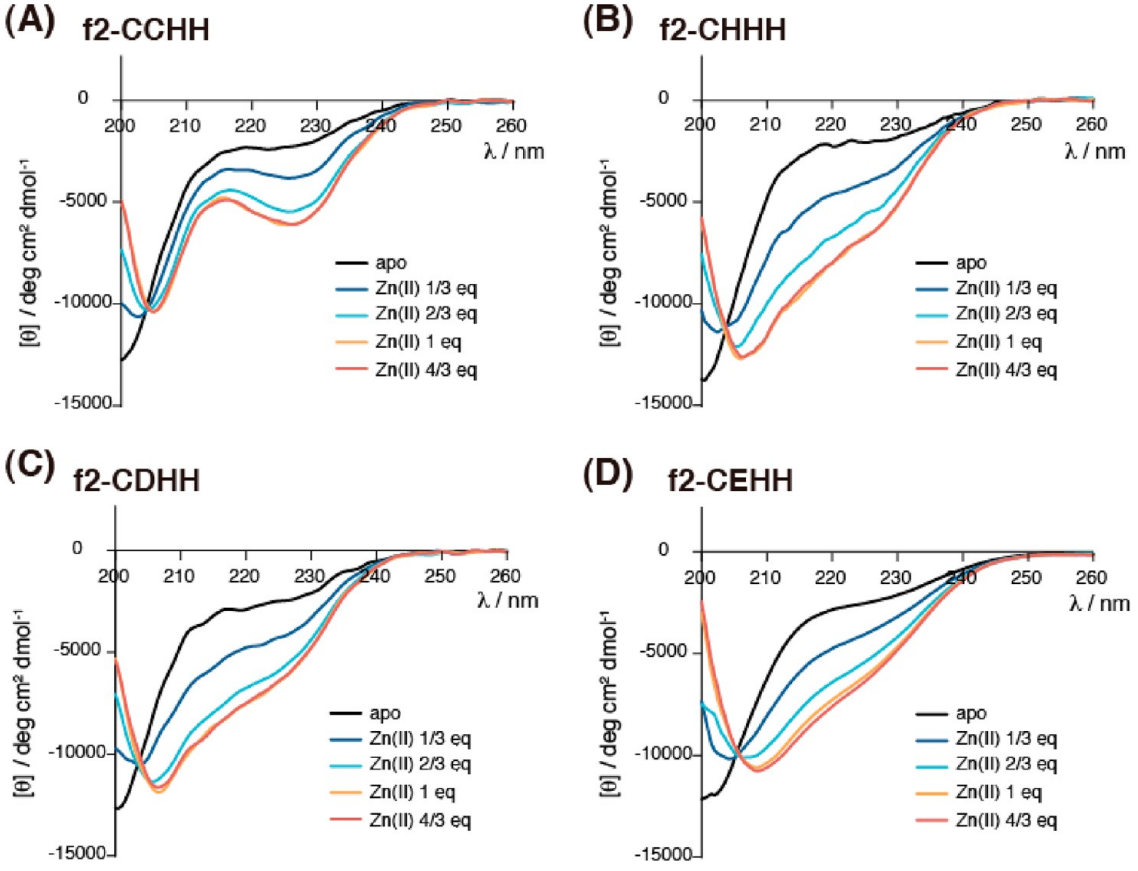


Figure 3. CD spectra of f2(CXHH) peptides (60 μM) in the absence and presence of $^{1}/_{3}$, $^{2}/_{3}$, 1, and $^{4}/_{3}$ equiv (20, 40, 60, and 80 μM, respectively) of $ZnSO_4$: (A) f2(CCHH), (B) f2(CHHH), (C) f2(CDHH), and (D) f2(CEHH).

results indicate that the f2(CCHH) and f2(CHHH) peptides have the typical tetrahedral Co(II) coordination geometry. The binding of Zn(II) to these peptides was shown by the disappearance of absorption bands when a 2-fold excess of Zn(II) ion compared with Co(II) was added (Figure 2A,B). On the other hand, f2(CDHH) and f2(CEHH) showed small LMCT and d−d absorption bands, suggesting that the Co(II) coordination geometry of f2(CDHH) and f2(CEHH) is

dx.doi.org/10.1021/bi300236m | *Biochemistry* 2012, 51, 3342−3348

different from that of f2(CCHH) and f2(CHHH). However, the fact that the d−d absorption bands disappeared upon addition of a 2-fold excess of Zn(II) ion compared with Co(II) indicated that a Co(II) ion was substituted with the spectroscopically inert Zn(II) ion (Figure 2C,D).

Binding of Zn(II) to the ligand-substituted zinc finger peptides was also confirmed by CD spectra showing Zn(II)-dependent conformational changes (Figure 3). In the absence of Zn(II), all of the peptides exhibited a negative band near 200 nm, suggesting that the peptides are in a largely random coil conformation. The addition of Zn(II) induced negative molar ellipticity around 208 and 220−230 nm, characteristic of a zinc finger structure containing an α-helix and a β-sheet. The CD spectral changes were saturated by the addition of equimolar Zn(II) concentrations to the peptides. These observations indicate that all of the peptides bind Zn(II) with a 1:1 stoichiometry and undergo a conformational change. This occurred despite the overall structures being different from that of f2(CCHH) (WT), as indicated by the CD spectra.

The binding affinity of f2(CXHH) for Zn(II) was determined by monitoring the zinc-dependent negative Cotton effect at 222 nm in the CD spectra under competitive conditions with ethylenediaminetetraacetic acid (EDTA) or ethylene glycol bis(2-aminoethyl ether)tetraacetic acid (EGTA). As shown in Figure 4 and Table 1, the Zn(II)

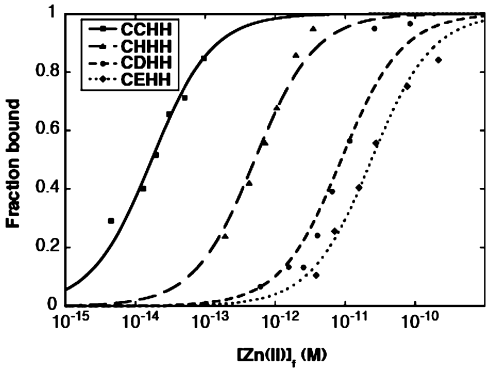


Figure 4. Zn(II) binding titration experiments for each finger 2 peptide. The calculated K_d values are listed in Table 1.

Table 1. Apparent Dissociation Constants for Binding between Zn(II) and the f2(CXHH) Peptides

peptide	K_d[a] (pM)
f2(CCHH)[4]	0.025 ± 0.0077
f2(CHHH)	0.40 ± 0.092
f2(CDHH)[4]	11 ± 4.5
f2(CEHH)	26 ± 13

[a] K_d values are averages of the results from three independent experiments ± the standard deviation.

dissociation constant of f2(CHHH) was 0.40 ± 0.092 pM, which is more than 10-fold weaker than that of wild type f2(CCHH) (0.025 ± 0.0077 pM)[4] but stronger than that of f2(CDHH) (11 ± 4.5 pM).[4] The Zn(II) dissociation constant of f2(CEHH) was 26 ± 13 pM, which is slightly weaker than that of f2(CDHH).

These results indicate that the f2(CXHH) (X = C, H, D, and E) peptides with metal-coordinated side chains positioned at the second conserved cysteine residue can fold by Zn(II) coordination. However, the substitution of ligand amino acids markedly changed the Zn(II) binding affinity [f2(CCHH) > f2(CHHH) > f2(CDHH) and f2(CEHH)] and the coordination geometry of a metal ion, especially in f2(CDHH) and f2(CEHH).

Thermal Stability of f2(CXHH) Peptides. To estimate the thermal stability of the ligand-substituted zinc finger peptides, CD spectra were measured at various temperatures between 20 and 80 °C (Figure 5). All the peptides exhibited a linear melting profile indicating weakly cooperative folding, consistent with our earlier results.[23−25] The WT peptide, f2(CCHH), showed no remarkable changes around 208 nm and only a slight decrease in ellipticity around 220−230 nm in a temperature-dependent manner. This indicates that the structure of f2(CCHH) is considerably stable, in agreement with a previous report.[23] Conversely, the ligand-substituted zinc finger peptides, f2(CHHH), f2(CDHH), and f2(CEHH), presented significant responses to temperature changes. The ellipticity was decreased across a broad range (200−240 nm), and the peak position of the negative Cotton effect observed at 208 nm and 20 °C was shifted to a shorter wavelength (~204 nm) with an increase in temperature. These results suggest that the overall structures of the ligand-substituted zinc finger peptides are deformed at high temperatures and that the thermal stabilities of the mutant peptides are lower than that of the WT peptide.

Measurements of DNA Binding Affinity of ZF3(CXHH). The DNA binding affinities of the CXHH-type (X = C, H, D, and E) zinc finger peptides were examined using ZF3(CXHH), in which the second zinc finger motif of the Zif268 zinc finger domain was substituted with the CXHH-type peptide[4] (Figure 1B). Figure 6 shows the results of EMSAs using FITC-labeled DNA fragments containing the target sequence of Zif268, 5′-GCG TGG GCG-3′, in the presence and absence of Zn(II). The equilibrium dissociation constants between ZF3(CXHH) and the target DNA in the presence of Zn(II) were determined by fitting the fraction of peptide-bound DNA to eq 1 (Table 2). Though ZF3(CDHH) and ZF3(CEHH) had lower affinities for DNA than WT ZF3(CCHH) in the presence of Zn(II), their dissociation constants remained in the range of 10^{-9} M, whereas the DNA binding affinity of ZF3(CHHH) was very low (3.5×10^{-7} M). The addition of 100 μM EDTA to the reaction mixtures yielded an inability of the ligand-substituted zinc finger proteins to bind DNA, whereas the WT maintained its binding to the target DNA (Figure 6, bottom). These results indicate that the binding of Zn(II) by peptides is necessary for the ligand-substituted zinc finger proteins to bind the target DNA. In addition, it suggests that the ligand-substituted derivatives are more sensitive to a decrease in Zn(II) concentration than is the WT, as expected from the low Zn(II) binding affinities. The order of DNA binding affinities (CCHH > CDHH > CEHH ≫ CHHH) was independent of the metal binding affinities (CCHH > CHHH > CDHH and CEHH) or the metal binding geometry. Previous reports indicated that the bulkiness of the conserved hydrophobic residues could influence DNA binding by a CCHH-type zinc finger domain derived from the GAGA transcription factor.[24,25] The DNA binding affinity of the ligand-substituted derivatives may correlate with differences in whole zinc finger structures depending on the bulkiness of the ligands.

Evaluation of Sequence Preferences of ZF3(CXHH). The crystal structure of the WT zinc finger domain of Zif268 with DNA shows that Zif268 binds to the 5′-GCG TGG GCG-3′ sequence. Each finger specifically recognizes a DNA triplet,

dx.doi.org/10.1021/bi300236m | Biochemistry 2012, 51, 3342−3348

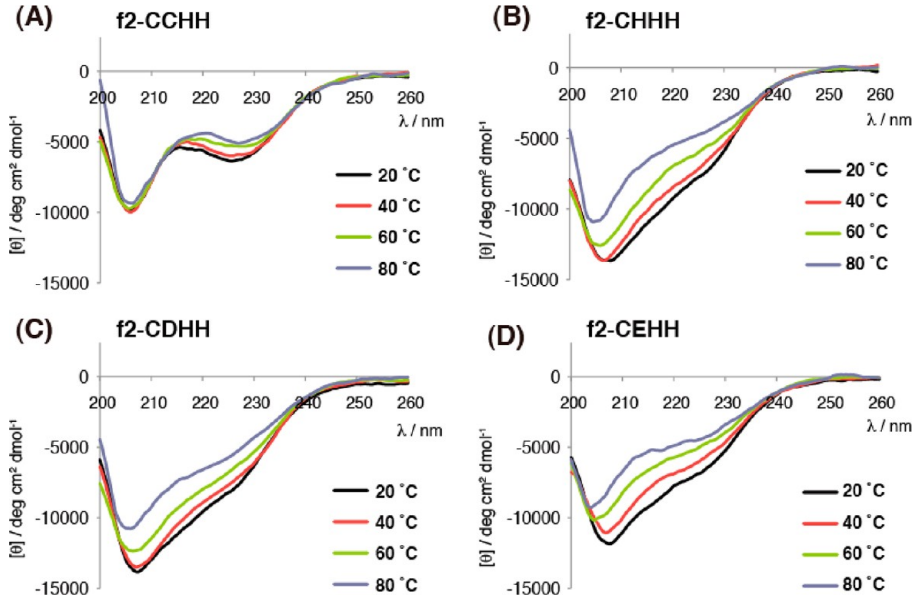


Figure 5. Temperature-dependent CD spectra of f2(CXHH) peptides (30 μM) in the presence of 100 μM Zn(II) between 20 and 80 °C: (A) f2(CCHH), (B) f2(CHHH), (C) f2(CDHH), and (D) f2(CEHH).

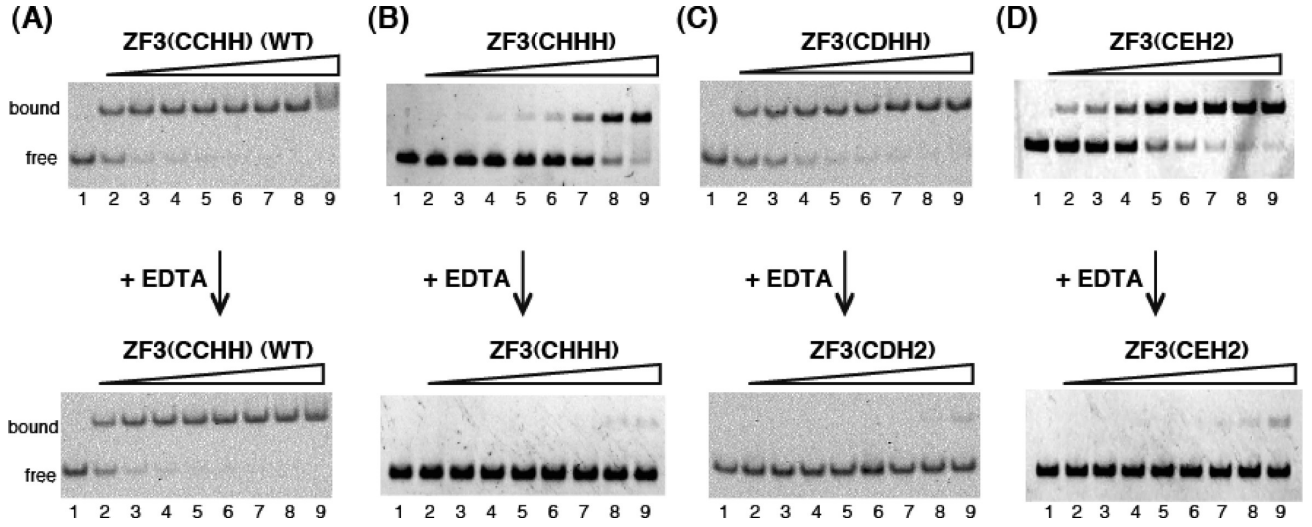


Figure 6. Gel mobility shift assay for ZF3(CXHH) proteins in the presence of 10 μM $ZnSO_4$ (top) and an additional 100 μM EDTA (bottom). The FITC-labeled dsDNA fragment containing 5′-GCG TGG GCG-3′ was used. Lanes 1−9 contained zinc finger concentrations of 0, 0.7, 2.1, 6.2, 19, 56, 167, 500, and 1500 nM, respectively: (A) ZF3(CCHH) (WT), (B) ZF3(CHHH), (C) ZF3(CDHH), and (D) ZF3(CEHH). The K_d values were calculated and are listed in Table 2.

using side chains of specific amino acid residues located on the recognition helix.[2,26,27] At the same time, selection and microarray experiments demonstrated that Zif268 can also bind to other DNA sequences, especially 5′-G-C-G-T/g-G/A-G-G-C/a/t-G-3′ (lowercase letters indicate bases selected less frequently).[28,29] There has yet to be a study identifying the relationship among DNA binding specificity, affinity, and metal binding properties. It is intriguing to investigate the effects of ligand substitution on DNA binding specificity. We prepared 64 kinds of dsDNA fragments, including 5′-GCG NNN GCG-3′ (N = A, C, G, and T; 4^3 = 64), in which the triplet subsite of the second finger (5′-TGG-3′ for the wild type) was substituted with NNN. EMSAs were performed for all 64 DNAs using a constant protein concentration, at which an ∼90% band shift was detected for the original target DNA (NNN = TGG). In agreement with previous reports,[28,29] ZF3(CCHH) bound most strongly to the NNN = TRG (R = G and A) sequences, followed by the GRG sequence. Weak DNA binding was observed in the lanes with (A/C)RG and TYG (Y = C and T) sequences (Figure 7A). The ligand-substituted zinc finger

Table 2. Equilibrium Dissociation Constants of ZF3(CXHH) and the Target DNA

peptide	K_d[a] (nM)
ZF3(CCHH)[4]	0.49 ± 0.09
ZF3(CHHH)	350 ± 45
ZF3(CDHH)[4]	2.1 ± 1.4
ZF3(CEHH)	9.8 ± 4.1

[a] K_d values are averages of the results from three independent experiments ± the standard deviation.

dx.doi.org/10.1021/bi300236m | Biochemistry 2012, 51, 3342−3348

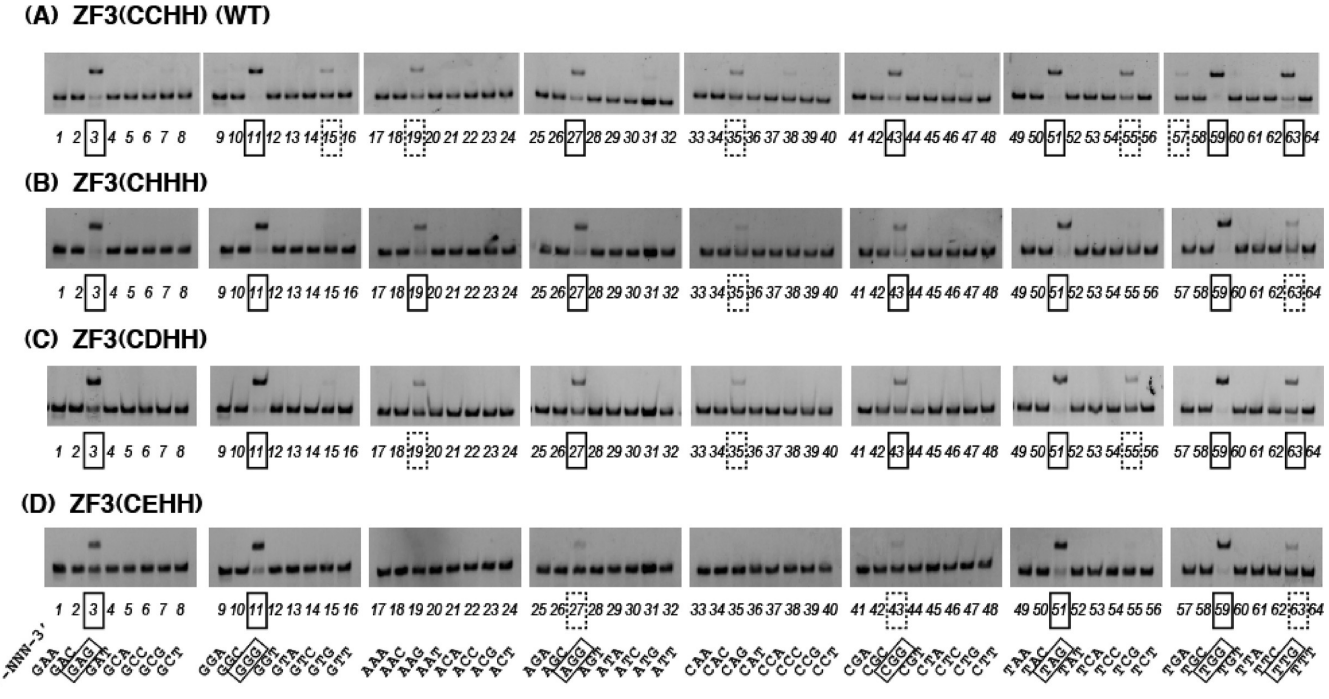


Figure 7. Examination of DNA binding sequence preferences of ZF3(CXHH) proteins. Results of EMSAs for 64 different DNA sequences containing 5′-GCG NNN GCG-3′ are shown. The NNN sequence for each lane (lanes 1−64) is indicated at the bottom of the figure. The concentrations of each peptide were set to allow detection of 90% band shifts for NNN = TGG (lane 59). The filled squares indicate the lanes showing strong DNA binding (>50% protein-bound fraction). The dotted squares indicate the lanes showing weak DNA binding: (A) ZF3(CCHH) (WT), (B) ZF3(CHHH), (C) ZF3(CDHH), and (D) ZF3(CEHH).

proteins, ZF3(CHHH), ZF3(CDHH), and ZF3(CEHH), presented a slightly limited, but mostly similar, DNA binding pattern compared with that of WT. Namely, they showed shifted bands for all NRG, with especially strong bands for TRG and GRG (Figure 7B−D). The contributions of fingers 1 and 3 to the binding specificity of the finger 2 subsite may not be negligible. In addition, EMSAs provide only crude and qualitative insight into the binding specificity of the WT and ligand-substituted zinc finger proteins. It is plausible, however, that the DNA binding specificity is well preserved among the ligand-substituted zinc fingers, at least in the context of a three-finger DNA binding domain. It has been suggested that DNA binding specificity is primarily determined by specific amino acid residues located at positions −1, 2, 3, and 6 on the recognition helix, based on the structure of Zif268 with DNA.[26,27] Our results using ligand-substituted zinc finger proteins also support the importance of these amino acids in DNA recognition.

In this study, we substituted a ligand cysteine residue of a zinc finger peptide with histidine, aspartic acid, or glutamic acid. It was determined that all peptides bind Zn(II) at a 1:1 stoichiometry, but that the binding affinity and coordination geometry of Zn(II) depend on the ligand residues. We also showed that all of the examined ligand-substituted zinc finger proteins retained the ability to bind DNA in the context of the full three-finger DNA binding domain in the presence of Zn(II). Of special interest is the fact that the DNA binding specificities of the ligand-substituted zinc finger proteins are almost the same as that of WT, despite differences in the metal binding characteristics, structural stability, and DNA binding affinity. Thus, zinc finger-type DNA-binding proteins with various DNA binding affinities that respond to a diverse range of Zn(II) concentrations can be created by substituting the Zn(II) ligand. Moreover, using the ScanProsite protein motif scanning tool (http://prosite.expasy.org/scanprosite/)[30] for the pattern [YF]-X-C-X(2,4)-[DEH]-X(3)-[YF]-X(5)-L-X(2)-H-X(3,5)-H indicates the existence of CXHH-type (X = D, E, and H) zinc finger motifs. Our study also contributes to an understanding of the biological significance of naturally occurring zinc finger motifs with atypical Zn(II) coordination sites.

AUTHOR INFORMATION

Corresponding Author

*Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan. Telephone: +81-774-38-3212. Fax: +81-774-32-3038. E-mail: imiki@scl.kyoto-u.ac.jp.

Funding

This work was supported in part by Grants-in-Aid for Young Scientists, and for the Global COE Program "International Center for Integrated Research and Advanced Education in Materials Science" from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank T. Morii for providing a DNA sequencer and Y. Azuma for helpful discussions.

ABBREVIATIONS

CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(2-aminoethyl ether)tetraacetic acid; FITC, fluorescein isothiocyanate; Fmoc, 9-fluorenylme-

thoxycarbonyl; Tris, tris(hydroxymethyl)aminomethane; UV−vis, ultraviolet−visible; WT, wild type.

REFERENCES

(1) Klug, A. (2010) The discovery of zinc fingers and their applications in gene regulation and genome manipulation. *Annu. Rev. Biochem. 79*, 213−231.

(2) Pavletich, N. P., and Pabo, C. O. (1991) Zinc finger-DNA recognition: Crystal structure of a Zif268-DNA complex at 2.1 Å. *Science 252*, 809−817.

(3) Frankel, A. D., Berg, J. M., and Pabo, C. O. (1987) Metal-dependent folding of a single zinc finger from transcription factor IIIA. *Proc. Natl. Acad. Sci. U.S.A. 84*, 4841−4845.

(4) Imanishi, M., Nakaya, T., Morisaki, T., Noshiro, D., Futaki, S., and Sugiura, Y. (2010) Metal-stimulated regulation of transcription by an artificial zinc-finger protein. *ChemBioChem 11*, 1653−1655.

(5) Rebar, E. J., and Pabo, C. O. (1994) Zinc finger phage: Affinity selection of fingers with new DNA-binding specificities. *Science 263*, 671−673.

(6) Beerli, R. R., Segal, D. J., Dreier, B., and Barbas, C. F., III (1998) Toward controlling gene expression at will: Specific regulation of the erbB-2/HER-2 promoter by using polydactyl zinc finger proteins constructed from modular building blocks. *Proc. Natl. Acad. Sci. U.S.A. 95*, 14628−14633.

(7) Segal, D. J., Dreier, B., Beerli, R. R., and Barbas, C. F., III. (1999) Toward controlling gene expression at will: Selection and design of zinc finger domains recognizing each of the 5′-GNN-3′ DNA target sequences. *Proc. Natl. Acad. Sci. U.S.A. 96*, 2758−2763.

(8) Maeder, M. L., Thibodeau-Beganny, S., Sander, J. D., Voytas, D. F., and Joung, J. K. (2009) Oligomerized pool engineering (OPEN): An 'open-source' protocol for making customized zinc-finger arrays. *Nat. Protoc. 4*, 1471−1501.

(9) Jamieson, A. C., Miller, J. C., and Pabo, C. O. (2003) Drug discovery with engineered zinc-finger proteins. *Nat. Rev. Drug Discovery 2*, 361−368.

(10) Blancafort, P., Segal, D. J., and Barbas, C. F., III (2004) Designing transcription factor architectures for drug discovery. *Mol. Pharmacol. 66*, 1361−1371.

(11) Patel, K., Kumar, A., and Durani, S. (2007) Analysis of the structural consensus of the zinc coordination centers of metalloprotein structures. *Biochim. Biophys. Acta 1774*, 1247−1253.

(12) Sousa, S. F., Lopes, A. B., Fernandes, P. A., and Ramos, M. J. (2009) The zinc proteome: A tale of stability and functionality. *Dalton Trans., 38*, 7946−7956.

(13) Green, A., and Sarkar, B. (1998) Alteration of zif268 zinc-finger motifs gives rise to non-native zinc-co-ordination sites but preserves wild-type DNA recognition. *Biochem. J. 333*, 85−90.

(14) Negi, S., Itazu, M., Imanishi, M., Nomura, A., and Sugiura, Y. (2004) Creation and characteristics of unnatural CysHis3-type zinc finger protein. *Biochem. Biophys. Res. Commun. 325*, 421−425.

(15) Nomura, A., and Sugiura, Y. (2002) Contribution of individual zinc ligands to metal binding and peptide folding of zinc finger peptides. *Inorg. Chem. 41*, 3693−3698.

(16) Simpson, R. J., Cram, E. D., Czolij, R., Matthews, J. M., Crossley, M., and Mackay, J. P. (2003) CCHX zinc finger derivatives retain the ability to bind Zn(II) and mediate protein-DNA interactions. *J. Biol. Chem. 278*, 28011−28018.

(17) Sénèque, O., and Latour, J. M. (2010) Coordination properties of zinc finger peptides revisited: Ligand competition studies reveal higher affinities for zinc and cobalt. *J. Am. Chem. Soc. 132*, 17760−17774.

(18) Morisaki, T., Imanishi, M., Futaki, S., and Sugiura, Y. (2008) Rapid transcriptional activity in vivo and slow DNA binding in vitro by an artificial multi-zinc finger protein. *Biochemistry 47*, 10171−10177.

(19) Giedroc, D. P., Keating, K. M., Williams, K. R., Konigsberg, W. H., and Coleman, J. E. (1986) Gene 32 protein, the single-stranded DNA binding protein from bacteriophage T4, is a zinc metalloprotein. *Proc. Natl. Acad. Sci. U.S.A. 83*, 8452−8456.

(20) May, S. W., and Kuo, J. Y. (1978) Preparation and properties of cobalt(II) rubredoxin. *Biochemistry 17*, 3333−3338.

(21) Vasák, M., Kägi, J. H., Holmquist, B., and Vallee, B. L. (1981) Spectral studies of cobalt(II)- and nickel(II)-metallothionein. *Biochemistry 20*, 6659−6664.

(22) Bertini, I., and Luchinat, C. (1984) High spin cobalt(II) as a probe for the investigation of metalloproteins. *Adv. Inorg. Biochem. 6*, 71−111.

(23) Negi, S., Imanishi, M., Sasaki, M., Tatsutani, K., Futaki, S., and Sugiura, Y. (2011) An arginine residue instead of a conserved leucine residue in the recognition helix of the finger 3 of Zif268 stabilizes the domain structure and mediates DNA binding. *Biochemistry 50*, 6266−6272.

(24) Dhanasekaran, M., Negi, S., Imanishi, M., Suzuki, M., and Sugiura, Y. (2008) Effects of bulkiness and hydrophobicity of an aliphatic amino acid in the recognition helix of the GAGA zinc finger on the stability of the hydrophobic core and DNA binding affinity. *Biochemistry 47*, 11717−11724.

(25) Dhanasekaran, M., Negi, S., Imanishi, M., and Sugiura, Y. (2007) DNA-binding ability of GAGA zinc finger depends on the nature of amino acids present in the *β*-hairpin. *Biochemistry 46*, 7506−7513.

(26) Elrod-Erickson, M., Benson, T. E., and Pabo, C. O. (1998) High-resolution structures of variant Zif268-DNA complexes: Implications for understanding zinc finger-DNA recognition. *Structure 6*, 451−464.

(27) Elrod-Erickson, M., and Pabo, C. O. (1999) Binding studies with mutants of Zif268. Contribution of individual side chains to binding affinity and specificity in the Zif268 zinc finger-DNA complex. *J. Biol. Chem. 274*, 19281−19285.

(28) Bulyk, M. L., Huang, X., Choo, Y., and Church, G. M. (2001) Exploring the DNA-binding specificities of zinc fingers with DNA microarrays. *Proc. Natl. Acad. Sci. U.S.A. 98*, 7158−7163.

(29) Swirnoff, A. H., and Milbrandt, J. (1995) DNA-binding specificity of NGFI-A and related zinc finger transcription factors. *Mol. Cell. Biol. 15*, 2275−2287.

(30) Sigrist, C. J., Cerutti, L., de Castro, E., Langendijk-Genevaux, P. S., Bulliard, V., Bairoch, A., and Hulo, N. (2010) PROSITE, a protein domain database for functional characterization and annotation. *Nucleic Acids Res. 38*, D161−D166.