

Selection of novel structural zinc sites from a random peptide library

Teruhiko Matsubara^{a,*}, Yuko Hiura^b, Osamu Kawahito^b, Mikito Yasuzawa^b,
Katsuhiro Kawashiro^b

^aDepartment of Biosciences and Informatics, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-8522, Japan

^bDepartment of Chemical Science and Technology, The University of Tokushima, 2-1 Minamijosanjima, Tokushima 770-8506, Japan

Received 17 September 2003; revised 28 October 2003; accepted 28 October 2003

First published online 12 November 2003

Edited by Hans Eklund

Abstract Zinc ion (Zn^{2+}) can be coordinated with four or three amino acid residues to stabilize a protein's structure or to form a catalytic active center. We used phage display selection of a dodecamer random peptide library with Zn^{2+} to identify structural zinc sites. The binding specificity for Zn^{2+} of selected sequences was confirmed using enzyme-linked immunosorbent and competitive inhibition assays. Circular dichroism spectra indicated that the interaction with Zn^{2+} induced a change in conformation, which means the peptide acts as a structural zinc site. Furthermore, a search of protein databases revealed that two selected sequences corresponded to parts of natural zinc sites of copper/zinc superoxide dismutase and zinc-containing ferredoxin. We demonstrated that Zn^{2+} -binding sequences selected from the random combinatorial library would be candidates for artificial structural zinc sites.

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Key words: Structural zinc; Random peptide library; Metalloenzyme; Phage display system; Sequence homology

1. Introduction

Zinc is contained in natural enzymes and proteins that are related to the transport of oxygen in respiration, electron transfer reactions for oxidoreduction, gene expression and catalytic reactions [1]. The zinc in proteins is classified as structural or catalytic according to coordination number and functions. Structural zinc is bound with four residues in a structural unit resembling a finger to regulate gene expression and to stabilize protein conformation in enzymes. In many cases, catalytic zinc is bound with three residues and one water molecule, and directly involved in enzymatic activity as Lewis acid [2,3].

Metal affinity domains and fragments of natural proteins have been used as molecular tools. For instance, a copper/nickel ion-binding tripeptide (Gly-Gly-His) was used for protein cross-linking, DNA/RNA cleavage and copper ion sensing [4–6]. Histidine-rich short peptides (histidine hexamer etc.)

have also been used to purify recombinant proteins. In the past decade, the peptide and protein display technique has been developed to obtain protein epitopes for target molecules from random combinatorial libraries [7,8]. This strategy has the potential of enabling metal-binding sites or catalytic sites of enzymes to be selected. Transition metal-binding peptides and antibodies have been selected from random libraries, and applied to immobilized metal affinity chromatography [9], the construction of metalloantibodies [10] and bioremediation [11,12]. However, these molecules were merely metal-containing sites and had no structural function. For de novo protein design, a scaffold like a zinc-finger domain is required for the specific binding of target ligands.

In the present study, a library of random dodecamer (12-mer) peptides was used in order to identify long affinity sequences [13]. We expected the selected peptides to induce conformational change due to metal-peptide interaction. Actually, the interaction between Zn^{2+} and one 12-mer peptide did cause a conformational change. The Zn^{2+} -peptide complex converged to a stable conformation, which was different from that of the peptide in the absence of Zn^{2+} . We describe novel sequences of structural zinc sites that were selected from the random peptide library. These sequences have the potential to act as a scaffold to support conformation of the recognition site of de novo protein.

2. Materials and methods

2.1. Reagents

A random 12-mer peptide library was purchased from New England BioLabs (Ph.D.-12 phage display peptide library kit, E8110S). The library (2.7×10^9 diversity) expresses peptides at the N-terminus of a minor coat protein III of filamentous bacteriophage with a Gly-Gly-Ser spacer.

2.2. Phage display selection

A chelating column (HiTrap Chelating, 1 ml, Amersham Pharmacia Biotech), which contains an iminodiacetic acid group linked to agarose beads, was used for the selection. The column was washed with 5–10 ml (5–10 column volumes) of distilled water and loaded with 0.5 ml of a 0.1 M solution of ZnCl_2 . The column was again washed with distilled water and equilibrated with a start buffer (0.02 M sodium phosphate, 0.5 M NaCl, pH 7.2). A phage solution containing 10^{10} plaque-forming units in 0.2 ml of the start buffer was applied to the column. After a wash with 5 ml of the start buffer to remove unbound phage particles, an elution buffer (0.05 M EDTA in the start buffer) was loaded. The eluate was placed in a centrifugal filter device (Centricon-30, Amicon) to remove the metal ions and then amplified by infection with *Escherichia coli* ER2738 (phage library kit). The amplified eluate was purified by precipitation with polyethylene glycol (PEG)/NaCl (PEG #6000, Nacalai Tesque) and used in the next cycle. After four rounds, individual phage clones were isolated. The phage

*Corresponding author. Fax: (81)-45-566 1447.

E-mail address: matsubara@bio.keio.ac.jp (T. Matsubara).

Abbreviations: ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; PBS, phosphate-buffered saline; Fmoc, 9-fluorenylmethoxycarbonyl; TFA, trifluoroacetic acid; IC₅₀, 50% inhibitory concentration; CD, circular dichroism; PDB, Protein Data Bank; SOD, superoxide dismutase

ssDNA was purified with a QIAprep Spin M13 kit (Qiagen) and used for DNA sequencing. The insert alignment was analyzed using a primer (5'-TAACACTGAGTTTCGTCACCAGTA-3') and a DNA sequencer (ABI Prism 310 genetic analyzer, PE Applied Biosystems) according to the instruction manual.

2.3. Enzyme-linked immunosorbent assay (ELISA)

The concentration of phage stock was estimated from the absorbance at 260 nm (when $A_{260} = 1$, [phage] = 1.1×10^{-8} M). The amplified phages were purified by precipitation with PEG/NaCl and diluted (0.1–1.0 nM) in phosphate-buffered saline (PBS, pH 7.4). A microtiter plate (MS-8796F, Sumilon) was blocked by incubating with 1% bovine serum albumin (BSA) in PBS for 2 h at room temperature. The plate was then washed with 0.5 M imidazole, 0.1 M EDTA, and distilled water [14,15]. A coupling reagent, 60 μ l of 0.5 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in PBS at pH 8, and 60 μ l of 5 mM *N*-(5-amino-1-carboxypentyl) iminodiacetic acid (Dojindo, Japan) in PBS at pH 8 were added to each well of the plate and incubated for 2 h at 37 °C. The plate was washed three times with 200 μ l of PBS containing 0.05% Tween 20 and then 100 μ l of 0.1 M metal solution (Zn^{2+} , Cu^{2+} , Co^{2+} , Ni^{2+} or Fe^{2+}) was added. After 15 min at room temperature, the plate was washed three times with PBS. The phage solution (50 μ l) was added to the metal ion-immobilized plate and incubated for 1 h at room temperature. The plate was washed three times with 200 μ l of PBS/0.2% Tween 20 and then an anti-fd bacteriophage antibody (B7786, Sigma) in 0.5% BSA/PBS was added. After 1 h at room temperature, the plate was washed three times with PBS. A peroxidase-conjugated anti-rabbit IgG antibody (A6154, Sigma) in 0.5% BSA/PBS was added and incubated for 1 h at room temperature. The plate was washed three times and a peroxidase substrate (*o*-phenylenediamine, 0.4 mg/ml) in citrate-phosphate buffer at pH 5.0 containing 0.02% (v/v) H_2O_2 was added. The plate was developed for 15 min and quenched with 1.5 M H_2SO_4 . Absorbance was measured at 492 nm.

2.4. Competitive inhibition assay

A serial dilution (0.01–0.1 M) of Zn^{2+} , Co^{2+} or Ni^{2+} containing 1 nM of phage in PBS was added to the Zn^{2+} -immobilized plate and incubated for 1 h at room temperature. The plate was washed and treated as described in Section 2.3. When the fraction (*f*) of phage binding to Zn^{2+} was plotted against the concentration of competing metal ion ($[M^{2+}]$), sigmoidal curves were obtained. The binding fraction was defined as $f = B/B_{max}$, where *B* is the binding of phages (absorbance at 492 nm), and B_{max} is the maximum binding of phages ($[M^{2+}] = 0$). Then $\log [f/(1-f)]$ was plotted against $\log [M^{2+}]$ and the IC_{50} (50% inhibitory concentration) was calculated from the intercept ($f = 0.5$) of the plots (data not shown).

2.5. Peptide synthesis

All peptide amides were prepared by solid-phase peptide synthesis on a 4-(2',4'-dimethoxyphenyl)-Fmoc-aminomethyl)-phenoxy resin (Fmoc-NH-SAL-Resin, Watanabe Chemical Industries) using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. After assembly of the sequence using *O*-benzotriazol-1-yl-*N,N,N'*-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazole hydrate/*N,N*-diisopropylethylamine (1:1:2) as coupling reagent, the peptide amides were cleaved from the resin with phenol/triisopropylsilane/1,2-ethanedithiol/trifluoroacetic acid (TFA) (2:2:1:33) for 6 h. After the solvent was removed, the resulting residue was washed with diethyl ether by decantation. All crude peptides were purified by reversed-phase high performance liquid chromatography (C_{18} column, 250 $\times$ 4.6 mm) with a linear gradient of water containing 0.1% TFA and acetonitrile containing 0.1% TFA at a flow rate of 0.5 ml/min. The major fractions were lyophilized and the peptide amides were finally characterized by matrix-assisted laser desorption/ionization/time-of-flight mass spectrometry using a matrix of sinapinic acid.

2.6. Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded on a Jasco J-820 spectropolarimeter using 0.1-mm cuvettes at a wavelength of 260–190 nm. A peptide stock solution in water was prepared and diluted to the desired concentrations in 5 mM NH_4OAc buffer (pH 7.5) containing 0.1–1.0 mM $Zn(OAc)_2$. The CD spectra are reported as mean residue ellipticity ($[\theta]$) in deg $cm^2/dmol$.

3. Results

3.1. Affinity selection with zinc ion

The 12-mer peptide library was a commercially available phage display system with a diversity of 2.7×10^9 . Divalent zinc ion (Zn^{2+}) was immobilized on agarose beads through an iminodiacetic acid chelating group. The phage library interacted with the immobilized Zn^{2+} , and then the beads were washed to remove unbound phages. The bound phages, together with Zn^{2+} , were stripped from the beads by adding 0.05 M EDTA. The eluate was amplified and this procedure was repeated four times. The relative yield of collected phages increased from 0.053% to 4.3% from the first to fourth round (data not shown). Then the enrichment of Zn^{2+} -binding phages was confirmed by ELISA with a Zn^{2+} -immobilized microtiter plate. The ELISA signal of fourth round phages

Table 1

Deduced amino acid sequences of peptides selected from the phage library using Zn^{2+} and their binding affinities for various metal ions

No.	Sequence ^a	Freq. ^b	Relative binding affinity ^c				
			Zn	Ni	Co	Cu	Fe
c04	<u>HYQH</u> NTTHPSRW	2	3.9 $\pm$ 0.7	3.4 $\pm$ 0.6	2.3 $\pm$ 0.7	1.5 $\pm$ 0.1	1.1 $\pm$ 0.1
c22	<u>HFQAQMR</u> HGHGH	1	3.6 $\pm$ 0.5	1.6 $\pm$ 0.1	2.1 $\pm$ 0.4	1.1 $\pm$ 0.0	0.9 $\pm$ 0.0
c20	<u>HQSHHYG</u> PRDHT	1	3.4 $\pm$ 0.4	2.6 $\pm$ 0.1	2.3 $\pm$ 0.2	1.3 $\pm$ 0.1	1.2 $\pm$ 0.1
c07	<u>HIKHH</u> PSSVPHA	1	3.2 $\pm$ 0.3	4.0 $\pm$ 0.3	3.7 $\pm$ 0.1	1.6 $\pm$ 0.1	1.7 $\pm$ 0.1
c03	<u>GSPHHNH</u> FKESH	1	3.0 $\pm$ 0.1	3.1 $\pm$ 0.2	0.4 $\pm$ 0.2	1.1 $\pm$ 0.1	1.1 $\pm$ 0.1
c09	<u>HSPHI</u> WSPHHGP	1	3.0 $\pm$ 0.3	3.0 $\pm$ 0.5	0.9 $\pm$ 0.2	1.4 $\pm$ 0.2	0.7 $\pm$ 0.1
c15	<u>HLQIPKPH</u> VHHT	1	2.9 $\pm$ 0.5	2.3 $\pm$ 0.6	1.2 $\pm$ 0.3	1.5 $\pm$ 0.2	1.0 $\pm$ 0.2
c18	<u>HSVHH</u> LPSPLSH	1	2.8 $\pm$ 0.2	2.1 $\pm$ 0.1	1.9 $\pm$ 0.3	1.3 $\pm$ 0.1	0.7 $\pm$ 0.0
c21	<u>HPHQAHP</u> SPKAH	1	2.5 $\pm$ 0.3	2.1 $\pm$ 0.2	0.9 $\pm$ 0.3	1.6 $\pm$ 0.3	1.4 $\pm$ 0.2
c01	<u>HSPLGSHH</u> HPKH	1	2.5 $\pm$ 0.2	2.6 $\pm$ 0.4	2.6 $\pm$ 0.5	1.3 $\pm$ 0.2	1.1 $\pm$ 0.1
c14	<u>HNHAHLPL</u> HPAP	1	2.5 $\pm$ 0.6	1.7 $\pm$ 0.4	0.0 $\pm$ 0.1	0.9 $\pm$ 0.0	1.1 $\pm$ 0.1
c11	<u>HNHHPH</u> STRQAS	1	2.4 $\pm$ 0.3	2.0 $\pm$ 0.6	0.2 $\pm$ 0.3	0.8 $\pm$ 0.1	1.2 $\pm$ 0.1
c10	<u>GTHVHH</u> PHSTST	1	2.4 $\pm$ 0.7	2.6 $\pm$ 0.2	0.2 $\pm$ 0.2	1.5 $\pm$ 0.3	0.9 $\pm$ 0.1
c02	<u>HMTELHH</u> SGVHQ	1	1.8 $\pm$ 0.4	2.3 $\pm$ 0.7	0.6 $\pm$ 0.2	1.2 $\pm$ 0.2	0.8 $\pm$ 0.0
Library	—	—	1.0 $\pm$ 0.1	1.0 $\pm$ 0.2	1.0 $\pm$ 0.3	1.0 $\pm$ 0.2	1.0 $\pm$ 0.1
c13	<u>HVHTH</u> PPQHRPP	1	0.5 $\pm$ 0.2	2.2 $\pm$ 0.2	0.2 $\pm$ 0.2	1.3 $\pm$ 0.1	1.2 $\pm$ 0.1

^aMetal ion-chelatable amino acids (His, Glu, and Asp) are underlined.

^bFrequency means number of times independently isolated from 23 plaques.

^cRelative binding affinity of phage at 1 nM for metal ions determined by ELISA. The absorbance ratio of each clone to the primary phage library was estimated.

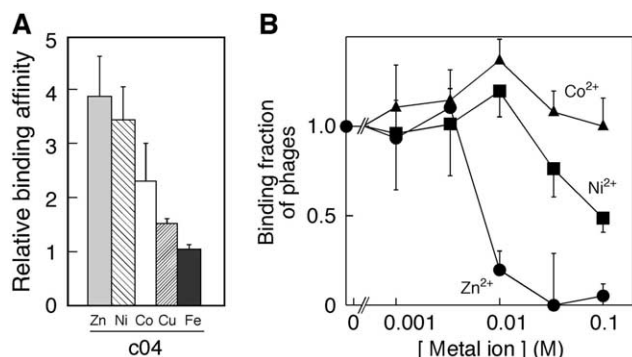


Fig. 1. A: Binding specificity for zinc of the selected phage clone c04. The relative binding affinity of c04 for metal ions (Zn^{2+} , Ni^{2+} , Co^{2+} , Cu^{2+} and Fe^{3+}) was determined by ELISA. B: Competitive inhibition assay of clone c04. The binding of c04 at 1 nM to the Zn^{2+} -immobilized plate was disrupted by increasing the concentration (0.001–0.1 M) of metal ion (Zn^{2+} , Ni^{2+} or Co^{2+}). The IC_{50} values for Zn^{2+} , Ni^{2+} , and Co^{2+} are 6.3 mM, 93 mM and > 100 mM, respectively. Each point is the mean for three wells. The standard deviation is indicated by an error bar.

showed simple saturation curves against phage concentrations; a 2.6-fold increase in binding affinity was observed at 1 nM (data not shown). After the fourth round of panning, 23 randomly selected individual phage clones were isolated. From the determination of binding affinity for Zn^{2+} at 1 nM by ELISA, 15 phage clones with a relative affinity ranging from 1.8 to 3.9 were screened (Table 1). DNA sequencing showed that 14 kinds of phage clones were obtained. All 14 peptide sequences contained four or five histidines, however, there was no obvious consensus in motif among the selected sequences.

3.2. Binding specificity of selected phage clones

Binding affinities of clones for metal ions (Ni^{2+} , Co^{2+} , Cu^{2+} and Fe^{3+}) were characterized by ELISA (Table 1). Since each of the clones had unique specificity characteristics, the order of binding affinity for metal ions was not correlated to that of the Irving–Williams series [16]. Binding affinities of the c04 phage clone, which had the highest affinity for zinc, followed the order of $\text{Zn}^{2+} > \text{Ni}^{2+} > \text{Co}^{2+} > \text{Cu}^{2+} > \text{Fe}^{3+}$ (Fig. 1A). Zn^{2+} , Ni^{2+} and Co^{2+} competed with the c04 phage clone to bind immobilized Zn^{2+} on the plate with an IC_{50} of 6.3 mM, 93 mM and > 100 mM, respectively (Fig. 1B). Results of the inhibition assay were consistent with the binding order of the c04 clone based on ELISA, indicating that the clone was

specific to zinc. In constant, clone c13 as a negative control had no affinity for metal ions except Ni^{2+} . Since most of the clones bound to Ni^{2+} , it appeared that all the selected sequences had universal affinity for nickel.

3.3. Peptide conformation and zinc titration

The c04 peptide was chemically synthesized and its secondary structure was determined by CD spectroscopy. A strong negative peak ($-11\,600\text{ deg cm}^2/\text{dmol}$) and a weak positive peak ($609\text{ deg cm}^2/\text{dmol}$) were observed at 198 and 225 nm, respectively, in 5 mM NH_4OAc (pH 7.5) (Fig. 2A). This suggested that the c04 peptide has no rigid structure. In the presence of Zn^{2+} , however, CD spectral change was observed. Both the negative and positive CD peaks weakened on the addition of Zn^{2+} , and the mean residue ellipticity at 200 or 220 nm was plotted against $[\text{Zn}^{2+}]/[\text{peptide}]$ (zinc titration, in Fig. 2B). The decrease of CD absorption at 198 nm was almost saturated at 1 equivalent of Zn^{2+} , and binding of stoichiometric Zn^{2+} to the peptide was confirmed.

Fig. 3 shows the difference in CD of the c04 peptide in the absence and presence of Zn^{2+} . The subtracted CD profile of the complex with a molar ratio of 1:1 had a positive peak at 190 nm and a negative peak at 200 nm. This profile is regarded as that of a β -turn structure [17]. At a molar ratio of metal to peptide of 10:1, another negative peak ($n\text{-}\pi^*$ transition) appeared at 225 nm. The magnitude of the $n\text{-}\pi^*$ peak is not quantitative, because the content of the β -form structure also contributes to the CD of $n\text{-}\pi^*$ transition. Therefore, we concluded that Zn^{2+} binding disrupted the β -turn structure of the peptide. Conformational change due to Zn^{2+} binding is often seen in structural zinc sites of naturally occurring proteins [16].

3.4. Sequence similarity search

Deduced amino acid alignments of the selected Zn^{2+} -binding peptides were compared with those of natural zinc proteins identified in databases such as SwissProt (Swiss Institute of Bioinformatics and Centre Médical Universitaire), PIR (Protein Information Resource), and PRF (Protein Research Foundation). We used BLAST [18] and FASTA [19] algorithms to search for sequence similarity. A FASTA search revealed that sequences at structural zinc sites of metalloproteins were similar to those of the selected peptides c03 and c20: copper/zinc superoxide dismutase (Cu/Zn-SOD) and zinc-containing ferredoxin (Zn-ferredoxin), respectively (Table 2). The zinc-containing sites were identified from X-ray crystal

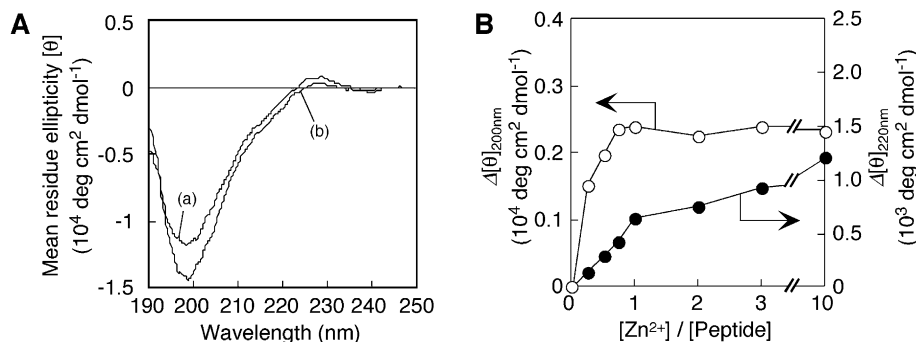


Fig. 2. A: CD spectra of peptide c04 as a function of Zn^{2+} concentration in 5 mM NH_4OAc at pH 7.5. (a) 0.1 mM peptide c04, (b) peptide c04 with 0.1 mM $\text{Zn}(\text{OAc})_2$. B: Zn^{2+} titration of peptide c04 monitored by CD change at 200 nm and 220 nm. Mean residue ellipticity at 200 (open circle) and 220 nm (filled circle) was plotted against $[\text{Zn}^{2+}]/[\text{peptide}]$.

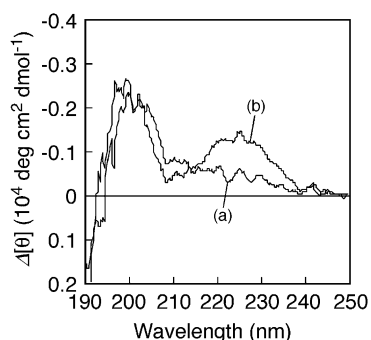


Fig. 3. Difference CD spectra of peptide c04. The spectrum of peptide with (a) 1 equivalent and (b) 10 equivalents of Zn^{2+} was subtracted from that of peptide without Zn^{2+} . A CD profile with negative peaks at 200 nm and 225 nm indicates a β -turn structure.

structures using the Protein Data Bank (PDB) [20]. Residues 3–12 of the c03 peptide showed homology to residues 62–71 of yeast Cu/Zn-SOD (PDB entry, 2JCW), where His-63 and His-71 coordinate with Zn^{2+} of its catalytic center. The c20 peptide was homologous with part of the Zn^{2+} -binding site (residues 16–26) of Zn-ferredoxin (1XER) as well. In both cases, sequences containing two out of four zinc ligands were obtained. In spite of polynuclear metalloproteins (Cu/Zn-SOD contains one copper and one zinc; Zn-ferredoxin contains one zinc and six irons), Zn^{2+} -binding sites were preferentially selected from the random library. Part of the catalytic zinc site of Mn-SOD was also similar to a c02 sequence, however, no binding affinity of the c02 phage clone for Mn ion was found by ELISA (data not shown). Sequences of four kinds of histidine-rich proteins were similar to c01, c03, c10, c11, c20 and c22 peptides. Since no structural analyses of the histidine-rich proteins have been made, the meaning of these matches is not known.

4. Discussion

In nature, structural zinc binds in a tetrahedral geometry to four metalloprotein residues such as His, Glu, Asp and Cys, whereas catalytic zinc binds to three residues [1,2]. Phage display technology has been developed to select structural zinc sites from a random peptide library. The peptide sequences selected in the present study bound specifically to the target Zn^{2+} (Table 1 and Fig. 1). The 14 peptide sequences selected contained four or five histidines, and the occurrence of histidine increased from 3.7% in the primary library to 33.9% (Table 1). Such histidine-rich peptides have also been obtained in studies with various divalent metal ions (Cu^{2+} , Cd^{2+} and Zn^{2+}) [9,11,12]. Moreover, the occurrence decreased for the remaining three chelating amino acids, Glu (2.2% to 1.2%),

Asp (3.1% to 0.6%) and Cys (0.6% to 0%). Unexpectedly, no cysteine was found in the sequences, although many structural zinc sites of the finger type have been found to have Cys-containing sequences [21]. The life cycle of phage particles in bacterial cells might influence this phenomenon, because it is assumed that the formation of a disulfide bond between the externally displayed peptides and a pIII coat protein disrupts the generation of phage virions or reduces the infectivity of phages [22]. Otherwise, elution with 0.05 M EDTA might not be good for the recovery of cysteine-containing phage clones.

The c04 peptide, having the highest affinity for Zn^{2+} , was chemically synthesized and its interaction with Zn^{2+} characterized. This peptide has four histidine residues. From the Zn^{2+} titration based on CD spectrometry, this peptide was found to interact with Zn^{2+} at a molar ratio of metal to peptide of 1:1 (Fig. 2B). However, there was no obvious consensus in motif among the selected sequences. These histidines might relate to the interaction, since this binding ratio (four histidines and one zinc ion) was in good agreement with that of structural sites in natural zinc proteins. The identification of coordination residues is now under way. Furthermore, structural change to the peptide was induced by the addition of Zn^{2+} . Difference CD spectra indicated that Zn^{2+} -peptide interaction disrupted the β -turn structure of the peptide and then a more stable Zn^{2+} -peptide complex formed (Fig. 3). However, interaction between a metal ion and His-rich peptide does not always induce conformational change [23]. The present study is the first report of the selection of a structural fragment containing a metal ion from a random library.

Significantly, a search of protein databases revealed that the selected peptide sequences showed homology to metal-binding sites of an oxidoreductase and electron transport protein (Table 2). The amino acid sequence of clone c03 showed a high degree of homology with part of the Zn-binding site of yeast Cu/Zn-SOD. In the catalytic center of Cu/Zn-SOD, the two metal ions Cu^{2+} and Zn^{2+} play important roles in the catalytic action. Zn^{2+} is bound with His-63, His-71, His-80 and Asp-83, where His-63 also coordinates with Cu^{2+} . It is known that Cu^{2+} interacts directly with superoxide anion substrates, and Zn^{2+} accelerates copper-substrate reactions and contributes to the protein's stability [16]. Two histidines contained in the c03 sequence (at positions 4 and 12) corresponded to His-63 and His-71 at the Zn-binding site in Cu/Zn-SOD, respectively. Since the remaining His-80 and Asp-83 in the SOD are separated from these histidines (63 and 71), it appears that only the c03 sequence was selected. This tendency was also seen in the relation between Zn-ferredoxin and the c20 sequence [24]. It is noteworthy that sequences of the selected peptides were sensitive to the kind of metal ion. The sequence of c03 was similar to that of the Zn^{2+} site in SOD, but not to

Table 2
Sequence similarity between the selected peptides and proteins in databases searched using the FASTA algorithm

Source	Residues	Sequence	PDB entry
Peptide c03	3–12	P <u>H</u> N H F K E S H	
Cu/Zn-SOD (baker's yeast)	62–71	P <u>H</u> F N P F K K T H	2JCW (62–71) etc.
Peptide c20	1–10	H Q S H H – Y G P R D	
Zn-ferredoxin (<i>Sulfolobus</i>)	16–26	H Q G H K V Y G P V D	1XER (16–26)

Identical residues are indicated in bold, and proposed metal-binding residues based on the crystal structures of the Protein Data Bank (PDB) are underlined. Sequences contained two out of four zinc ligands.

that of the Cu²⁺ site. Similarly, although Zn-ferredoxin has six irons, the sequence of c20 corresponded to that of the Zn²⁺-binding site. Such selectivity is consistent with the Zn²⁺-specific affinity confirmed by ELISA (Table 1 and Fig. 1A). It therefore seems that a long sequence (more than 10 amino acids) is necessary to specifically bind metals. Kjaergaard et al. also selected zinc-binding peptides (9-, 18-, and 27-mer), but found no similarity in sequence with natural proteins [12].

We demonstrated that the peptides selected from a random peptide library acted as structural zinc sites and had a high degree of homology to parts of the zinc-containing sites of natural proteins. The selection of peptide-displaying systems has been performed with numerous targets, but few reports have discussed the conformation of the peptides selected [25]. In general, folding is required for a protein (peptide) molecule to have binding specificity and/or higher functions. For example, a cyclic peptide library that contains two Cys residues which form a disulfide bond has been used to compensate for enthalpy loss of interaction between the peptide and target molecule [26]. Functional domains often require the modification and manipulation of proteins, thus, the selected sequences obtained here can be used as structural units for maintaining conformation and switching molecular recognition for de novo protein design.

Acknowledgements: We thank Dr. T. Hohsaka (Okayama University) for DNA analysis. This work was supported by Grant-in-Aid for the Encouragement of Young Scientists 1478046 (T.M.) from the Ministry of Education, Culture, Sports, Science and Technology.

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