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Mass Spectrometric Determination of Zn^{2+} Binding/Dissociation Constant for Zinc Finger Peptides

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Abstract : In the present study, we proposed a simple ESI-MS model for determining Zn^{2+} binding (or dissociation) constants for zinc finger peptides (ZFPs) with a unique ββα fold consensus. The ionization efficiency (response) factors for this model, *i.e.*, α and β, could be determined for ZiCo ZFP with a known Zn^{2+} binding constant. We could determine the binding constants for other ZFPs assuming those with a ββα consensus conformation have the same α/β response ratio. In general, the ZPF dissociation constants exhibited K_d values of 10^{-7}~10^{-9} M, while K_d values for a negative control non-specific Zn^{2+} peptides were high, *e.g.*, 5.5×10^{-6} M and 4.3×10^{-4} M for BBA1 and melittin, respectively.

Key words : zinc finger, binding constant, electrospray-mass spectrometry, zinc ion

Introduction

Since its discovery in transcription factor IIIA (TFIIIA) from *Xenopuslaevis,* zinc finger proteins (ZFPs) have been extensively researched due to their implications for eukaryotic protein-nucleic acid interactions.[1-7] ZFPs are one of the most common DNA binding motifs and play important roles in gene regulation.[3] In particular, $Cys_2His_2(C_2H_2)$ represent the most studied zinc finger motifs. ZFPs acquire DNA binding structures from their unstructured form via Zn^{2+} complexation, *i.e.*, protein folding coupled with metal binding. The so-called zinc metallo-finger motif acquires a compact ββα form via the Cys-Zn^{2+}-Cys and His-Zn-His bridges.[6] The ββα fold unit constitutes a single zinc finger domain that can recognize 3 to 4 DNA base pairs in a DNA major groove.[7] DNA base recognition uses the interactions of amino acid side chains located in the recognition α-helix. Extended DNA recognition is also possible via the tandem repeating of zinc finger domains.

Mass spectrometry (MS) has been used widely to study metal ions interactions with oligonucleotides, peptides, and proteins.[8-17] For zinc fingers, a variety of MS experiments have been used to study, for example, the stoichiometry for zinc ion transfer from metallothionein to zinc finger peptides, Zn^{2+}chelation by consensus zinc-finger arrays, the Zn^{2+} binding sites, and relative affinity for other transition metals.[18-22] Recently, our group showed electrospray ionization-mass spectrometry (ESI-MS) can be used to study noncovalent complexes formed between a zinc-finger peptide and DNA double helix.[23,24] In addition, it was found that the detected noncovalent complexes reflected the binding properties between these two interacting partners.

Because Zn^{2+} binding is crucially important to zinc fingers in inducing protein folding that ensures specific DNA base sequences are recognized, the Zn^{2+} binding (or dissociation) constants of zinc fingers are important information for studying zinc fingers. Zinc ion binding constants of zinc fingers are traditionally determined via UV spectroscopy. Despite UV method being widely used, it involves a back titration, which is time-consuming and requires significant sample amounts for analysis.

In contrast, ESI-MS, which is a fast analysis with low sample consumption, has been used extensively to determine the binding constants for various non-covalent complexes

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formed in solution.[25-33] In this study, we proposed an ESI-MS based titration method for measuring the Zn^{2+} binding constants of various natural and artificial zinc finger proteins.

Experimental

Materials

All of the peptides used in this study were custom synthesized via Fmoc-based solid-phase method (Peptron Inc., Daejeon, Korea) and purified using a Prominence LC-20AB RP-HPLC (Shimadzu, Kyoto, Japan). Table 1 shows the peptides and amino acid sequences being studied. These peptides are natural or designed with a C_2H_2 structure and generally contain a repeated amino acid sequence, *i.e*., the conserved sequence for Y/F-X-C-X(2 to 4)-C-XXX-Y/F-XXXXX-L-XX-H-X(3 to 5)-H with zinc ion-specific binding properties. The ammonium hydrogen carbonate (NH_4HCO_3) used to buffer the solution and zinc acetate ($Zn(CH_3COO)_2$) used as a zinc ion source were purchased from Sigma (Seoul, Korea) and used without further purification. The peptides and zinc acetate were prepared as a stock solution in 10 mM NH_3HCO_3 buffer solution (pH 7.5).

Mass Spectrometry

ESI mass spectrometry was performed using an ion trap mass spectrometer (LCQ Deca, Thermo, San Jose, CA, USA). The mass spectrometry parameters were set as follows: positive mode, +3.5 kV needle voltage, 110°C capillary temperature (note, a low temperature was used to avoid thermally denaturing the zinc finger peptides), +10 V capillary voltage, +20 V tube lens offset voltage, AGC off, *m/z* 200~2,000 mass range. The ion injection time was carefully adjusted at approximately 5 ms so that the experiments were done below the space charge limit of the ion trap. The prepared sample solutions were infused directly with a flow rate of 3 μL/min using a syringe pump built into the mass spectrometer.

Table 1. Peptides and their primary sequences

Peptides	Primary sequences ($NH_2 \rightarrow$ COOH)
Sp11	QHI**C**HIQG**C**GKVYGKTSHLRA**H**LRW**H**TGER
Sp13	KFA**C**PE**C**PKRFMRSDHLSK**H**IKT**H**QNKK
CF2II4	PYT**C**SY**C**GKSFTQSNTLKQ**H**TRI**H**TGEK
CF2II6	PYT**C**PY**C**DKRFTQRSALTV**H**TTKL**H**PL
Sp1HM1	QHI**C**HIQG**C**GKVYGQSNTLKQ**H**TRI**H**TGER
Sp1HM3	KFA**C**PE**C**PKRFMQRSALTV**H**TTKL**H**PNKK
ZiCo	YI**H**AL**H**RKAFAKIARLER**H**IRALE**H**AA
BBA1	YTVPSATFSRSDELAKLLRLHAG
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ

Sp11 and Sp13: the first and third finger, respectively, of Sp1, a human RNA polymerase II specific transcription factor with a sequence preference for the GC box.
CF2II4 and CF2-II-6: the fourth and sixth finger, respectively, of a *Drosophila* transcription factor with a preference for the AT-rich element.
Sp1HM1: chimeric mutant of Sp11 and CF2II4.
Sp1HM3: chimeric mutant of Sp11 and CF2II4 with specificity for AT-rich sequence.
ZiCo: an artificially designed peptide with a classical zinc finger motif and coil-coil structure.
BBA1: an artificially designed peptide with a ââá fold in a Zn^{2+} free form.
Melittin: a peptide with no Zn^{2+} binding affinity as a negative control.

Titration equation

We used the following titration model based on basic thermodynamic equations to determine the peptide-Zn^{2+} binding/dissociation constants for zinc-binding peptides.

$$P + Zn^{2+} \rightarrow P{:}Zn^{2+} \quad (1)$$

where P represents a peptide.

For the peptide-Zn^{2+} binding reaction **1**, the binding/dissociation constant (K_b or K_d) can be calculated from Equation **2**.

$$K_b = \frac{[P{:}Zn^{2+}]}{[P][Zn^{2+}]} = \frac{1}{K_d} \quad (2)$$

where [P] and [Zn^{2+}] are the concentration of a free peptide and Zn^{2+}, respectively, and [P:Zn^{2+}] represents the Zn^{2+}-bound peptide concentration. During the ESI-MS experiments, [P] and [P:Zn^{2+}] were determined by measuring the ion intensities for the corresponding species. However, because the peptides and Zn^{2+}-bound peptides exhibited different ionization efficiencies, the following equations were suggested.

$$I_P = \alpha[P] \quad (3)$$

$$I_{P:Zn^{2+}} = \beta[P{:}Zn^{2+}] \quad (4)$$

where α and β are the ionization efficiency (response) parameters for peptide P and P:Zn^{2+} metal-bound complexes, respectively. The following mass balance equation was established for the Zn^{+} concentration.

$$[Zn^{2+}] = [Zn^{2+}]_0 - [P{:}Zn^{2+}] \quad (5)$$

Where $[Zn^{2+}]_0$ is the initial Zn^{2+} concentration. The above equations yield Equation **6**.

$$K_b = \frac{\alpha I_{P:Zn^{2+}}}{I_P\{\beta[Zn^{2+}]_0 - I_{P:Zn^{2+}}\}} \text{ or}$$

$$K_d = \frac{\beta I_P[Zn^{2+}]_0}{\alpha I_{P:Zn^{2+}}} - \frac{I_P}{\alpha} \quad (6)$$

Futher,

$$\beta = \frac{(\alpha + K_b I_P) I_{P:Zn^{2+}}}{K_b [Zn^{2+}]_0 I_P} = \frac{(\alpha + K_d I_P) I_{P:Zn^{2+}}}{[Zn^{2+}]_0 I_P} \quad (7)$$

The response factor, α, can be estimated from independent calibration experiments by measuring the ion intensities of P as a function of the peptide concentration. Furthermore, once α is determined, Equation **7** yields the ionization response factor, β, for the Zn^{2+}-bound peptides, *i.e.*, $P:Zn^{2+}$, for a reference zinc-finger peptide with a known Zn^{2+}-binding constant by measuring the ion intensities of its free and Zn^{2+}-bound peptides (I_P and $I_{P:Zn^{2+}}$) at different initial Zn^{2+} concentration, $[Zn^{2+}]_0$. Once the α/β ratio for a ZFP with a known zinc ion binding constant (K_b) is determined, the β value for ZFPs with unknown K_b values can be obtained by assuming the α/β ratio is more or less constant for all ZFPs, which is the only assumption used in this study. Because all ZFPs have similar compact ββα structural motifs, this assumption appears reasonable.

Results and Discussion

Mass spectra of zinc-binding peptides

To determine the K_b or K_d values from the mass spectrometric results for zinc-binding peptides, the solvent

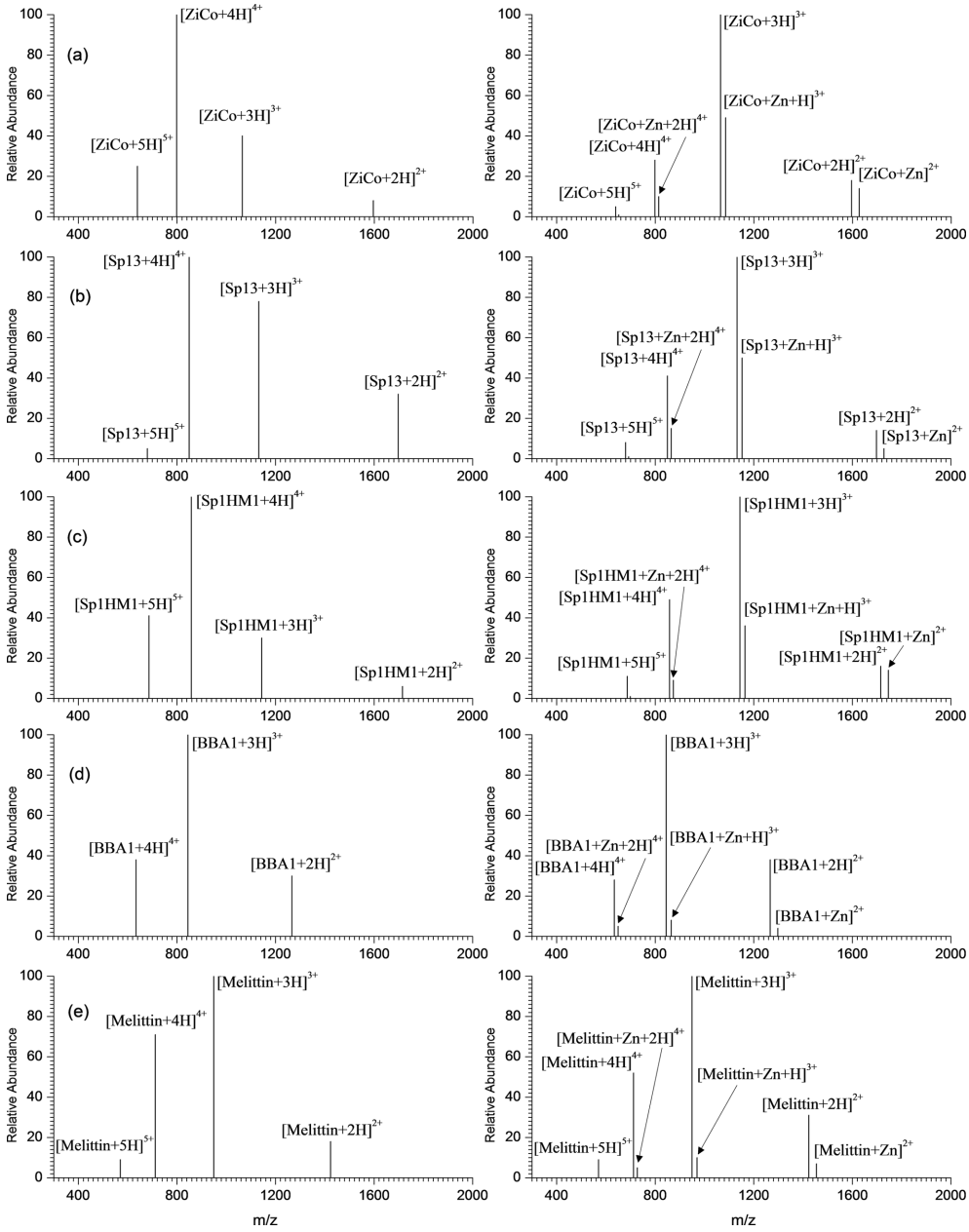

Figure 1. ESI full mass spectra with(right) and without (left) Zn^{2+} addition.(a) ZiCo, (b) Sp13, (c) Sp1HM1, (d), BBA1 and (e) melittin. Conditions: ammonium acetate buffer, pH 7.5; 20 μM equimolar concentration of the peptide and Zn^{2+}.

pH and mass spectrometer cone voltage were optimized to favor folded ZFP conformations. For example, the ESI solution pH must be maintained at ~7.5, which is similar to the native environment.

Figure 1 shows the ESI mass spectra obtained using 20 ìM ZFP peptides alone (left panel) and with an equimolar peptide and Zn^{2+} mixture (right panel). The charge-state envelope generally shifted to lower charge states, when the peptide:Zn^{2+} complexes formed via Zn^{2+} addition. For example, ZiCo peptide (Figure 1a) under zinc free conditions exhibited +2 to +5 charges with the +4 charge state being most abundant. Adding zinc ions in an equimolar ratio caused the noncovalent peptide:Zn^{2+} complexes to dominate with a +3 charge state, while the +5 charge state was negligible. Similar charge-state shifts were observed for other zinc specific binding peptides: Figure 1b, Sp13; Figure 1c, Sp1HM1 [also including Sp11, CF2II4, and CF2II6 (spectra not shown)]. The same experiments were performed using other non-zinc ion binding peptides such as BBA1 and melittin with similar molecular masses, ~3 kDa, to the other ZFPs as a control (see Figure 1d and e). The charge-state shift after Zn^{2+} binding did not occur for these peptides. Furthermore, Zn^{2+} binding did occur to any insignificant degree compared to the ZFP peptides.

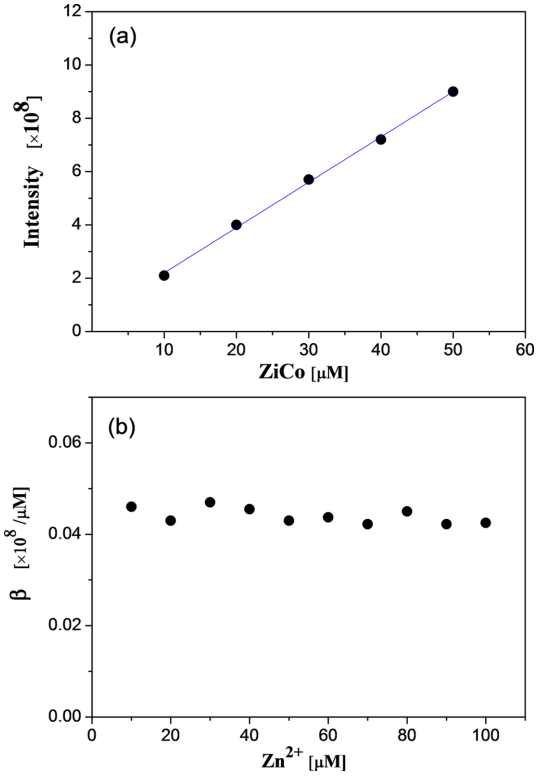

Figure 2. (a) The ion peak intensity of ZiCo peptide versus ZiCo concentration (10~50 μM); the slope yields response factor α. (b) Response factor β, for ZiCo. The Zn^{2+} concentration varied from 10~100 μM.

The above charge-state shift can be understood as the peptide folding after binding to Zn^{2+}. Without Zn^{2+}, the ZFPs tend to exhibit unfolded conformations. However, taking Zn^{2+} into a Zn^{2+} binding site formed the ZFP's specific folded ββα structure, which lowered the overall charge-state distribution.

The ratio of ionization response factors, α/β

Here, we used ZiCo, whose Zn^{2+} dissociation constant was reported as $(4\pm1)\times10^{-6}$ based on isothermal titration calorimetry (ITC)[35], acted as a reference peptide for determining the response factor ratio, *i.e.*, α/β. First, the MS peak intensities were measured at ZiCo concentrations from 10 to 50 μM without adding Zn^{2+} to determine α (see Figure 2a). All of the observed charge states were considered when calculating the MS peak intensities. The response factor, α, for ZiCo was estimated from this experiment as 0.17×10^8/μM ($R^2 = 0.99$) using Equation **3**. The α values for the other peptides were similarly determined ($R^2 \geq 0.99$) and are shown in Table 2.

Second, the zinc-free and zinc-binding ZiCo ion peak intensities were measured by adding Zn^{2+} at 10~100 μM to the 20 μM ZiCo solution. The zinc-free and zinc-binding ZiCo ion peak intensities from the ESI full mass spectrum for each initial Zn^{2+} concentration, *i.e.*, $[Zn^{2+}]_0$, were measured to determine I_p and $I_{p:Zn}{}^{2+}$, respectively. The response factor, β, for the zinc-binding ZiCo could be determined for each $[Zn^{2+}]_0$ using the literature K_d value for ZiCo and Equation **7**. The β value was generally constant over the measured $[Zn^{2+}]_0$ range and averaged $\beta = 0.044(\pm0.002)\times10^8$/μM (see Figure 2b). These experimental results yielded an ionization response factor ratio, α/β, of 3.86.

Zn^{2+} binding/dissociation constant determination

Based on the assumption that all ZFPs have the same α/β ratio, the response factors, β, for other ZFPs could be determined as shown in Table 2.

Furthermore, to determine the Zn^{2+} binding/dissociation constants, the full ESI mass spectra were obtained for 20 μM equimolar Zn^{2+}:peptide mixed solution of the other ZFP peptides. The β value and Equation **6** could be used to calculate the Zn^{2+} binding/dissociation constants for a variety of ZFPs (see Table 3). In general, ZFPs exhibited dissociation constants from 10^{-7}~10^{-9}. Specifically, Sp13

Table 2. The response factors α and β [$\times10^8$/μM]

Peptide	α	β	Peptide	α	β
ZiCo	0.17	0.044	Sp1HM1	0.24	0.062
Sp11	0.21	0.054	Sp1HM3	0.38	0.098
Sp13	0.23	0.060	BBA1	0.36	0.093
CF2II4	0.33	0.085	Melittin	0.29	0.075
CF2II6	0.31	0.080			

Table 3. The dissociation constants obtained in this study

Peptide	Dissociation constants, K_d
ZiCo	$(4\pm1) \times 10^{-6\#}$
Sp11	$(6.2\pm0.5) \times 10^{-8}$
Sp13	$(7.2\pm1.1) \times 10^{-9}$
CF2II4	$(5.9\pm0.7) \times 10^{-7}$
CF2II6	$(5.5\pm1.2) \times 10^{-7}$
Sp1HM1	$(6.6\pm0.9) \times 10^{-8}$
Sp1HM3	$(4.8\pm0.4) \times 10^{-8}$
BBA1	$(5.5\pm1.2) \times 10^{-6}$
Melittin	$(4.3\pm0.8) \times 10^{-4}$

#:reference value: $\alpha/\beta = 3.86$

exhibited the lowest dissociation constant, 7.2×10^{-9}, Sp1HM1/Sp1HM3 exhibited a moderate dissociation constant, $\sim10^{-8}$, and CF2II4/CF2II6 exhibited the highest dissociation constant, $\sim10^{-7}$. Non-specific peptides, such as BBA1 and melittin, exhibited large dissociation constants, 5.5×10^{-6} and 4.3×10^{-4}, respectively, which is consistent with their Zn^{2+} non-specificity. Furthermore, the dissociation constant for Sp13 determined here is in the same order of magnitude of the literature value, *i.e*., $(2.8\pm0.9)\times10^{-9}$.[36]

Conclusions

We propose an ESI-MS based method to determine the Zn^{2+} binding/dissociation constants for zinc finger peptides, which have conserved amino acid sequences and C_2H_2 zinc finger motif structures. By determining the ion efficiency parameters for zinc free peptides (α) and Zn^{2+}-peptide complexes (β) for a reference ZFP, *i.e*., ZiCo, we could readily determine the binding (or dissociation) constants for other ZFPs, for example, Sp11, Sp13, CF2II4, CF2II, Sp1HM1, and Sp1HM3. Our proposed method assumed the response factor ratio, α/β, was the same for all ZFPs with a $\beta\beta\alpha$ zinc-finger motif conformation. Although not described above, we performed a comparative study using the so-called De Pauw's method,[37] which agreed well the Zn^{2+} dissociation constants obtained via our proposed method. Whether this approach can be used for zinc-binding peptides with different conformations is currently questionable. However, our model is useful for determining Zn^{2+} binding (or dissociation) constants for zinc-binding peptide analogues with similar structural motifs, and should be further applicable to other metal ion binding constant determinations.

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