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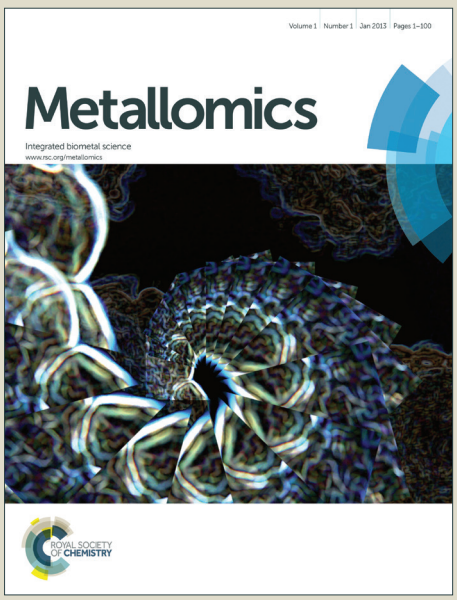

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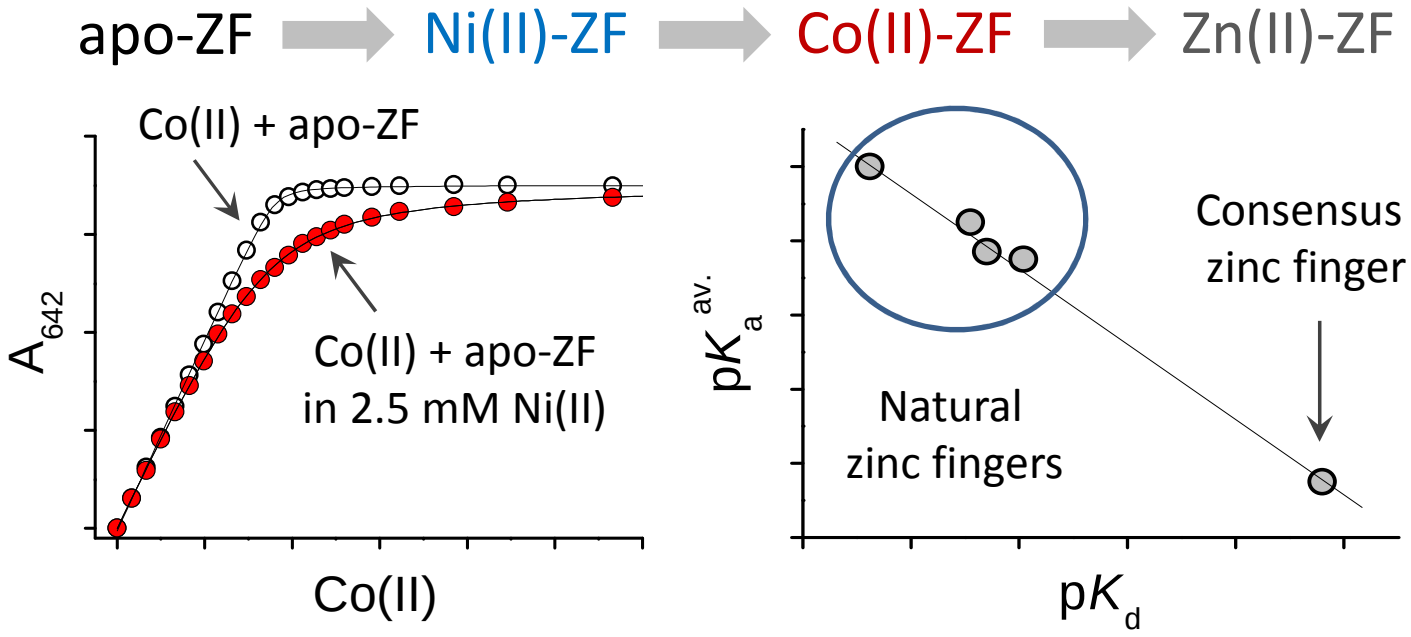

Metal binding properties of the zinc finger metallome – insights into variations in stability

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Abstract

Zinc is one of the most widespread metal ions found in biology. Of the expected 3000 zinc proteins in the human proteome, most contain zinc in structural sites. Among these structures, the most important are zinc fingers, which are well suited to facilitate interactions with DNA, RNA, proteins and lipid molecules. Knowledge regarding their stability is a critical issue in understanding the function of zinc fingers and their reactivity under fluxing cellular Zn(II) availability and different redox states. Zinc stability constants that have been determined using a variety of methods demonstrate wide diversity. Recent studies on the stability of consensus zinc fingers have demonstrated that the known metal-ion affinities for zinc fingers may have been underestimated by as much as three or more orders of magnitude. Here, using four natural ββα zinc fingers, we compare in detail several different methods that have been used for the determination of zinc finger stability constants, such as common reverse-titration, potentiometry, competition with metal chelators, and a new approach based on a three-step spectrophotometric titration. We discuss why the stabilities of zinc fingers that are determined spectrophotometrically are frequently underestimated due to the lack of effective equilibrium competition, which leads to large errors during the processing of the titration data. The literature stability constants of many natural zinc fingers have been underestimated, and they are significantly lower when compared with the consensus peptides. Our data show that in the cell, some naturally occurring zinc fingers may potentially be unoccupied and are instead loaded transiently with Zn(II). Large variations in stability within the same class of zinc fingers have demonstrated that the thermodynamic effects hidden in the sequence and structure are the key elements responsible for the differentiation of the stability of the zinc finger metallome.

Introduction

Among all of the transition metal ions in biology, zinc is the most widespread.[1] It has been shown that aside from its catalytic and structural role in proteins, it is also transiently present in proteins, where it specifically functions under zinc (formally Zn(II)) fluxes and buffering conditions.[2] Of the expected 3000 zinc proteins in the human proteome, most contain Zn(II) within structural sites.[3] Among these structures, the most important are zinc fingers, small domains in which the coordination of Zn(II) allows the folding of relatively short stretches of polypeptide chains into well-defined 3D structures that are well suited to facilitate protein interactions with other domains of the same or a different protein, or macromolecules such as DNA, RNA, and lipid molecules.[4] Apart from a few exceptions, the Zn(II) in zinc fingers is generally bound by histidine (H) and cysteine (C) residues in a tetrahedral geometry. Of the three types of coordination, i.e., CCHH (ZnS_2N_2), CCCH (ZnS_3N) and CCCC (ZnS_4) in zinc fingers, the first is the most common due to its presence in classical zinc fingers with a ββα-like structure, which constitute the majority of proteins, particularly transcription factors.[5, 6]

Zinc finger domains serve various functions. Because they are present in different types of proteins with different structural binding modes, their affinity for Zn(II) varies. The literature data show that the dissociation constants of zinc fingers vary from submicromolar to attomolar values. Their affinities have been determined under various conditions using different methodologies.[7-14] Such a wide range of affinities suggests that the metal affinity can be a factor that controls zinc finger functionality in terms of the Zn(II) availability in the cell, where free Zn(II) concentrations vary from 10^{-9} to 10^{-11} M.[2, 15] It is believed that weakly bound Zn(II) results in zinc fingers that are not fully occupied with zinc under physiological conditions. Another explanation for the wide variation in zinc finger stability in the literature could be the use of various methods that result in different stability constants for similar or even the same zinc fingers.[16, 17]

Zn(II) is commonly called a "spectroscopically silent" metal ion. The number of physicochemical methods that can be used in stability studies is limited, and they frequently rely on indirect techniques. The most common method for the determination of the affinity constants of zinc fingers relies on direct Co(II) titration into the peptide, followed by a displacement of the Co(II) with Zn(II) (reverse Zn(II)-titration). Co(II) is isostructural with Zn(II), and its complex with a zinc finger peptide has a number of characteristic bands, both in the UV and Vis range. These complexes allow quantitative control of the competition equilibria between Zn-ZF and Co-ZF (Zn(II) and Co(II)-loaded zinc finger peptides).[8, 12, 18] Other methods that are used less frequently are based on fluorescence, protein dialysis, pH-metry (potentiometry), isothermal titration calorimetry, and competition with other proteins or small chelators.[13, 19-22] Recent studies on the consensus zinc finger peptide CP1 and other natural and non-natural Zn(II)-binding motifs have determined the affinities when in competition with metal ion chelators and showed that zinc fingers (single and double) form much more stable complexes with Co(II) and Zn(II) than previously reported.[13, 19, 23] The dissociation constants determined by the competition were found to be three or more orders of magnitude lower than was previously determined spectrophotometrically.

In this report, using four different natural ββα zinc fingers we compared several methods for determining zinc finger dissociation constants, such as classical reverse-titration, potentiometry (pH-metry), competition with metal chelators and a new approach that requires a three-step titration with Ni(II), Co(II) and Zn(II). Our goal is to show the limitations of each method, especially spectroscopically, in determining zinc finger stability constants, as these methods frequently lead to significantly different stability constant values. We also focus on the reasons for the significant differences in stability between naturally occurring zinc fingers and commonly studied consensus models. Finally, we address the issue of the biological consequence of the variations in stability of the zinc finger metallome.

Experimental

Materials

Anisole, thioanisole, HEPES, EDT, acetic anhydride (Ac_2O), ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA), *N*-(2-hydroxyethyl)ethylenediamine-*N*,*N*′,*N*′-triacetic acid (HEDTA), ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), Chelex 100, Tris base, $NiCl_2 \cdot 6H_2O$, $HClO_4$, potassium hydrogen phthalate (KHP), and a standard solution of 0.1 M NaOH were purchased from Sigma-Aldrich. Acetonitrile (ACN), $NaClO_4 \cdot H_2O$, $Co(NO_3)_2 \cdot 6H_2O$ were obtained from Merck. NaCl, ethyl ether (Et_2O), KNO_3, HNO_3 and analytical weights of $ZnSO_4 \cdot 7H_2O$, were purchased from POCH (Gliwice, Poland). *N,N*-Dimethylformamide (DMF), dichloromethane (DCM), 1-methyl-2-pyrrolidinone (NMP), *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), trifluoroacetic acid (TFA), *N,N*-diisopropylethylamine (DIEA), piperidine, TentaGel R Ram and Fmoc-protected amino acids were obtained from Iris Biotech GmbH (Marktredwitz, Germany). The exact concentrations of each of the metal salts were confirmed by a representative series of ICP-MS measurements.[23, 24] All of the pH buffers used in this study were treated with Chelex-100 resin to eliminate any metal-ion contamination.

Peptide synthesis

The MTF1-1, Sp1-3, ZF133-11 and ZF278-1 zinc finger peptides were synthesized via solid phase synthesis using the Fmoc strategy on a TentaGel R Ram Amide Rink (substitution 0.2 mmol/g) and a Liberty 1 microwave-assisted synthesizer (CEM). The reagents excess, cleavage and purification were performed as previously described.[25] The acetylation of the N-terminus was performed using acetic anhydrate in the presence of DIEA. The N-terminal acetylated resin-attached peptides were cleaved from the resin with a mixture of

TFA/thioanisole/EDT/anisole (90/5/3/2 v/v/v/v) over a period of 2 h, followed by precipitation in cold (-20°C) Et_2O. The crude peptide pellets were collected by centrifugation, dried and then purified via HPLC (Dionex Ultimate 3000) on Phenomenex C18 columns using 0.1% TFA with an ACN gradient. The purified peptides were identified by ESI mass spectrometry with an API 2000 Applied Biosystems instrument. The identified and calculated mass values are listed in the captions of Figures S2-S5 (*Supporting Information*).

Potentiometric titration

The protonation constants of the ZF133-11 zinc finger peptide and the stability constants of its Zn(II) and Co(II) complexes in the presence of 4 mM HNO_3 and 96 mM KNO_3 (I = 0.1 M) were determined at 25°C using pH-metric titration over a range of 2.5 to 10.8 (Molspin automatic titrator, Molspin) using standardized 0.1 M NaOH as a titrant. An accurate concentration of NaOH was determined by the titration of a 4.0 mM standard solution of potassium hydrogen phthalate prepared directly before the measurement. Changes in the pH were monitored with a combined glass-Ag/AgCl electrode (Biotrode, Methrom). Sample volumes of 1.7-2.0 ml, a ZF133-11 concentration of 300 μM and employed metal/ligand ratios of 2:1-1.1:1 were used. The data were analyzed using the SUPERQUAD program.[26] The ionic product of the water used in the data processing was 13.80, which represents a 0.1 M ionic strength.[27]

Equilibration of the zinc fingers in the metal buffers

Zinc finger peptides at a concentration of 50 μM were equilibrated in different 1.0 mM chelator-Zn(II) metal buffers over 24 h. The set of 1 mM EDTA, HEDTA and EGTA metal buffers was prepared in 50 mM HEPES buffer, pH 7.4, with 100 mM NaCl and 0.05 - 0.95 mM $ZnSO_4$ to obtain a pZn ($-\log[Zn(II)]_{free}$) with a range of 9.80 to 14.89 (Tables S1-S3). TCEP, a Zn(II) non-binding disulfide reducing agent, was used in the equilibration to protect

the thiols.[28] The samples were measured in a 2-mm quartz cuvette on a Jasco J-815 spectropolarimeter at 25°C. The temperature was controlled by a Peltier heating/cooling system. Five accumulations were averaged using a 5-nm band width, a 100-nm/min scanning speed, and a 1.0-nm data pitch. Due to the high absorbance of the metal buffer components, the CD spectra that were recorded in the range of 210 to 265 nm with an ellipticity at 215 or 220 nm wavelengths were taken for the zinc finger peptide saturation analysis. In the other CD measurements of the Zn-ZF complexes, a range of 190 to 265 nm was used. The amount of Zn(II) transferred from the metal buffer component to the zinc finger peptide was considered during the re-calculation of the final pZn values. Tables S1-S3 present the pZn values, both before and after each peptide equilibration. All of the pZn calculations were calculated based on the published protonation and Zn(II) stability constants of EDTA (β_{HL} = 10.17, $\beta_{\mathrm{H_2L}}$ = 16.28, $\beta_{\mathrm{H_3L}}$ = 18.96, $\beta_{\mathrm{H_4L}}$ = 20.96, $\beta_{\mathrm{H_5L}}$ = 22.47, $\beta_{\mathrm{ZnHL}}^{\mathrm{EDTA}}$ = 19.44, $\beta_{\mathrm{ZnL}}^{\mathrm{EDTA}}$ = 16.44), HEDTA (β_{HL} = 9.81, $\beta_{\mathrm{H_2L}}$ = 15.18, $\beta_{\mathrm{H_3L}}$ = 17.78, $\beta_{\mathrm{ZnL}}^{\mathrm{HEDTA}}$ =14.6) and EGTA (β_{HL} = 9.40, $\beta_{\mathrm{H_2L}}$ = 18.18, $\beta_{\mathrm{ZnL}}^{\mathrm{EGTA}}$ = 12.60) and were performed using MINEQL 4.6 software.[29, 30] All of the experimental points for each zinc finger peptide were fitted with Hill's equation.

pH-titration of the zinc finger peptides

The pH-dependent formation of the zinc finger complexes was performed using CD measurements. For that purpose, 50 μM zinc finger peptide solutions containing 60 μM of Zn(II) were prepared in 0.1 M $NaClO_4$ acidified to pH ~ 2 and then titrated with 0.1 M NaOH in a pH range from ~2 to ~8. No TCEP was used due to the occurrence of weak thiol oxidation in the acidic pH. The samples were measured in a 2-mm quartz cuvette on a Jasco J-815 spectropolarimeter at 25°C. The temperature was controlled by a Peltier heating/cooling system. Three accumulations from 190-265 nm were averaged using a 5-nm band width, a 100-nm/min scanning speed, and a 1.0-nm data pitch. The ellipticity at 215 or 220 nm was

taken for the determination of the average pK_a values for each zinc finger complex. The data were fitted with Hill's equation.[19] It should be noted that the spectra obtained at pH 7.4 were identical to those obtained in the equilibration experiments with the chelators, although the spectral range differed in each case.

Spectroscopic titrations

All of the spectroscopic measurements were recorded on a Jasco V-650 spectrophotometer in a 1-cm quartz cuvette. The experiments were performed in chelexed 50 mM HEPES buffer with 100 mM NaCl or $NaClO_4$ in the presence of 250 μM of the non-binding metal ion disulfide reducing agent TCEP.[28] Either 35 μM or 3.5 μM of the zinc finger peptides were used for the experiments. The binding of Ni(II) and Co(II) to the peptides was monitored at 400 and 642 nm, respectively. The binding of Zn(II) to the peptide was monitored in the reverse titration by a decrease in the Co-ZF characteristic absorbance at 642 nm. The experimental titration points of the zinc finger peptides with the Ni(II) were processed with Equation 3, in order to obtain the K_d value of Ni-ZF. The effective affinity of the Co(II) to the peptides was determined by a zinc finger peptide titration with Co(II) in the presence of various Ni(II) concentrations (0-2500 μM). The dissociation constants were calculated by processing the experimental points with Equation 7. This procedure requires the fixation of the initial total Ni(II) concentration and the K_d value of Ni-ZF as constants. Similar to above, the dissociation constants of Zn-ZF were determined by a Zn(II) titration of the peptide in the presence of 100-3500 μM of Co(II). Because the absorbance at 642 nm decreased during the experiment, the final experimental points were reflected in such a way as to present the increases in the absorbance and peptide saturation. This modification is required for the proper use of Equation 7. Because of this, the initial Co(II) concentration and the Co-ZF

dissociation constant obtained from the above Ni(II)/Co(II) competition were fixed as constant values.

Results and discussion

Equations

The determination of the dissociation constant (K_d) of a metal-ligand complex (ML) requires knowledge regarding the concentrations of the reactants ([M] and [L]) that are in equilibrium (Eq. 1).

$$K_d = \frac{[M][L]}{[ML]} \quad (1)$$

The status of the metal ion (M) bound to its ligand (L) is analyzed by the measurement of the absorbance (A) at a characteristic complex wavelength found at the point with the largest change in absorbance between the minimal (A_{min}) and maximal (A_{max}) values. Rearrangement of the equilibrium concentrations of the reactants to their total values in Equation 1 allows one to process an entire set of spectroscopic titration points using Equations 2 or 3, where c_M and c_L are the total metal and ligand concentrations, respectively. A similar approach allows us to use Equation 3 for other techniques; however, the A values must be replaced by a characteristic measured parameter, such as fluorescence intensity or ellipticity.

$$[ML] = \frac{K_d + c_M + c_L - \sqrt{(K_d + c_M + c_L)^2 - 4c_L c_M}}{2} \quad (2)$$

$$A = A_{min} - \left(\frac{A_{min} - A_{max}}{2c_L}\right)\left(K_d + c_M + c_L - \sqrt{(K_d + c_M + c_L)^2 - 4c_L c_M}\right) \quad (3)$$

If the ligand molecule competes with more than one metal ion (M1 and M2), additional equilibria must be taken into consideration (Eq. 4 and 5), which might be combined into one equilibrium equation (Eq. 6) where M1L and M2L correspond to the ligand complexes with M1 and M2, and K_d^{M1} and K_d^{M2} are their dissociation constants (*Supporting Information*).

$$K_d^{\mathrm{M1}} = \frac{[\mathrm{M1}][\mathrm{L}]}{[\mathrm{M1L}]} \quad (4)$$

$$K_d^{\mathrm{M2}} = \frac{[\mathrm{M2}][\mathrm{L}]}{[\mathrm{M2L}]} \quad (5)$$

$$[\mathrm{M2L}] = \frac{[\mathrm{M2}][\mathrm{M1L}]K_d^{\mathrm{M1}}}{[\mathrm{M1}]K_d^{\mathrm{M2}}} \quad (6)$$

Equation 6 can be converted to an absorbance-dependent mode (Eq. 7), similar to Equation 3, by using the minimal, maximal and actual absorbance values (*Supporting Information*). One must note that the value of A corresponds to the absorbance of the M1L complex when it competes with M2. Equation 6 is ideal for calculating the reverse-titration of the M1-ZF complex with M2, by using the absorbance decrease that quantitatively corresponds to the decreasing concentration of the M1-ZF complex. One condition that is necessary for the application of Equation 7 to the spectrophotometric titration is removing the absorbance of the M2-ZF complex at the chosen wavelength.

$$\mathrm{A} = \mathrm{A_{min}} - \left(\frac{\mathrm{A_{min}} - \mathrm{A_{max}}}{2\mathrm{c_L}\left(K_{\mathrm{d}}^{\mathrm{M1}} - K_{\mathrm{d}}^{\mathrm{M2}}\right)}\right)\left(\mathrm{c_{M2}}K_{\mathrm{d}}^{\mathrm{M1}} + \mathrm{c_L}K_{\mathrm{d}}^{\mathrm{M1}} + \mathrm{c_{M1}}K_{\mathrm{d}}^{\mathrm{M2}} - \mathrm{c_L}K_{\mathrm{d}}^{\mathrm{M2}} - \sqrt{\left(\mathrm{c_{M2}}K_{\mathrm{d}}^{\mathrm{M1}} + \mathrm{c_L}K_{\mathrm{d}}^{\mathrm{M1}} + \mathrm{c_{M1}}K_{\mathrm{d}}^{\mathrm{M2}} - \mathrm{c_L}K_{\mathrm{d}}^{\mathrm{M2}}\right)^2 - 4\mathrm{c_{M2}}\mathrm{c_L}K_{\mathrm{d}}^{\mathrm{M1}}\left(K_{\mathrm{d}}^{\mathrm{M1}} - K_{\mathrm{d}}^{\mathrm{M2}}\right)}\right) \quad (7)$$

The details of the derivations of Equations 2, 3 and 7 are presented in the *Supporting information*. We have also provided formatted linear equations that are ready to copy-and-paste and be used in any software that allows experimental data processing (*Supporting Information*).

ββα zinc fingers

Many of the experimental studies performed on classical zinc fingers were completed using consensus zinc fingers (CP1), first introduced by Berg *et al.* in 1991.[31] The CP1 zinc finger sequences are based on a certain number of zinc finger sequences that were known at the time and include the majority of the critical amino acid residues responsible for the formation of stabilizing interactions, such as the hydrophobic core (Figure 1a).[31-33] To avoid the accumulation of all of the intramolecular interactions that are present in CP1 zinc fingers, but not necessarily all that are present in a natural zinc finger sequence, we chose four naturally occurring ββα zinc fingers for use in this study. Two of the zinc fingers, MTF1-1 (first zinc finger of human MTF1 factor)[18] and Sp1-3 (third zinc finger of human Sp1 factor),[16,17] have been extensively studied. The other two, ZF278-1 (first zinc finger of ZF278 protein) and ZF133-11 (11-th zinc finger of ZF133 protein), are less well known (Figure 1b). The last zinc finger (ZF133-11) was chosen to have the lowest number of amino acid residues with acid-base properties in order to be used in potentiometric studies. Because our aim is to compare the different methods used for the determination of the dissociation constants of zinc fingers, we have applied different experimental approaches for the selected zinc finger peptides. To indicate differences in the constant values obtained by the different methods, K_d^* refers to a value that is not comparable with the appropriate K_d value.

Experimental results

To estimate the affinities of Zn(II) for the zinc fingers in a classical reverse-titration method, all of the zinc finger peptides were directly titrated with Co(II) and the spectrophotometric titrations were fitted into Equation 3. Table 1 presents the apparent K_d^* values of the Co-ZF complexes that were determined for the two different peptide concentrations. The values obtained using 3.5 μM of the peptides are ~1 order of magnitude lower when compared to the

values determined using 35 μM of the zinc finger peptides. The reverse titrations of Co-ZF with Zn(II) using Equation 7 give a pK_d^* of 8.3 for Sp1-3 and 10.2 for ZF278-1 (Table 1). The values of the Zn(II) complexes with MTF-1 and Sp1-3 are comparable to those that have been previously published, where the dissociation constants were determined using the same reverse-titration method.[8, 12, 16-18]

The application of different methods for determining affinity based on the competition of a second ligand with comparable peptide affinity for a particular metal ion demonstrates that dissociation constants can be up to several orders of magnitude lower than those obtained using a classical reverse-titration.[11-13, 19, 25, 34] Metal ion chelators are convenient and inexpensive Zn(II) competitors and therefore are frequently used in competition experiments. Because their absorption in the UV range tends to be high, they are typically not used for spectrophotometric titrations. Instead, the application of spectropolarimetry allows one to use metal ion chelators in the near-UV range (low energy) to monitor the conformational changes of zinc finger peptides that are associated with Zn(II) coordination.[13, 19] All of the peptides used in this study were equilibrated with a set of metal buffers containing EGTA, HEDTA and EDTA to maintain the free Zn(II) [$Zn(II)_{free}$] within a range of ~10^{-10} to 10^{-15} M. Changes in the ellipticity presented as a function of -log[$Zn(II)_{free}$] allow the use of a one-binding-site model. Figure 2a presents the isotherms of all of the zinc fingers saturated with Zn(II) and includes the appropriate curve overlays. The zinc finger K_d values that were determined with this method are three-four orders of magnitude lower than those obtained from the reverse-titration and vary between 2.4×10^{-12} M for MTF-1 and 9.1×10^{-14} M for ZF278-1 (Table 1). These values are not comparable with the previously published values, but they are in the femtomolar range, which has been observed for other zinc fingers that were characterized using the same competition method.[13, 19, 34]

To increase the number of applicable methods for studying zinc finger affinity, we also used potentiometry. This method is frequently used for low-molecular-weight molecules (including peptides); however, its precise usage is limited to molecules with a relatively low number of acid-base groups that are, preferably, chemically different. Because ββα zinc finger peptides are ~30 amino acid motifs containing four binding motifs (His and Cys) and a variable number of other dissociating residues, they are not good subjects for potentiometric studies. Our choice of the ZF133-11 zinc finger was dictated by the fact that this peptide, aside from its metal binding residues, possesses only two Glu and one Lys residue. Table 2 presents both the cumulative and step dissociation constants of the ZF133-11 zinc finger and its complexes with Zn(II) and Co(II). All of the formed metal-ligand complexes are 1:1 with a variable number of associated protons. Figure 3 demonstrates the species distribution of the Co(II) and Zn(II) complexes in a wide range of pHs. The complex MH_3L^+, which is present at low pH values, corresponds to a peptide with a metal ion bound by two His resides; the remaining binding groups are protonated.[13] The MHL^- and ML^{2-} complexes are functional zinc fingers (ZnS_2N_2) either with or without a protonated ε-amine group on the Lys residue. $MH_{-1}L^{3-}$ corresponds more closely to a complex with one deprotonated water molecule bound to a metal ion.[35] The apparent dissociation (pK_d) constants of the Zn-ZF and Co-ZF complexes at pH 7.4, calculated for pH 7.4 based on the data from Table 1, are 12.48 and 8.38, respectively (Table 1). This value is very similar to the one obtained from the chelator competition but is significantly different from the value determined spectrophotometrically in the classical reverse-titration.

Due to the inconsistency in the affinity results obtained using the above methods, we decided to apply a new approach based on a modification of the reverse-titration method. Because both of the Co(II) and Zn(II) dissociation constants determined potentiometrically differ from the spectroscopic ones, and the affinity of Zn(II) for a zinc finger, as determined

by the reverse-titration method, depends on the initially determined Co-ZF stability, it became clear that an erroneous value for Co-ZF would significantly affect the final value. All of the spectroscopic titrations presented in Figure 4 demonstrate a sharp curve, which suggest that the Co-ZF dissociation constants may be overestimated. To solve this issue we introduced an additional step to the reverse-titration method, which involves an initial titration of the peptide with Ni(II) and an independent titration of the Ni-ZF with Co(II). The last step remains the same and relies on the reverse-titration of Co-ZF with Zn(II). It is well known that Ni(II) forms less-stable complexes with zinc fingers than Co(II) and Zn(II).[7, 17] An example of the ZF133-11 titration is presented in Figure 5, and it demonstrates a micromolar affinity for Ni(II) (Table 1). The shape of this titration curve and the peptide fractional saturation below 80% exclude the possibility of overestimation of the dissociation constant value. All of the other peptides demonstrated similar affinities; however, the Ni-MTF1-1 complex was found to have the highest K_d value, 21 μM (Table 1).

The titration of the Ni-ZF complexes with Co(II) exhibits different shaped curves depending on the amount of excess Ni(II) (Figure 6). The dissociation constant values (K_d^*) obtained with the different Ni(II) concentrations (0 - 2.5 mM) vary significantly; however, a characteristic plateau is observed in titrations with a high excess of Ni(II) (inset of Figure 6b). This clearly demonstrates that the determined dissociation constant (K_d^*) of the Co-ZF complex is significantly overestimated in a direct peptide titration, even at lower peptide concentrations. The data in Table 1 show that both values vary by 0.5 to 2.5 orders of magnitude, although the difference between the values becomes more pronounced the higher the Co(II) affinity becomes for the peptide. Similarly, the pK_d^* value of Zn-ZF also depends on the excess of Co(II), which in this study ranged from 0.1 - 3.5 mM (Figure 6c). Titration of all of the zinc finger peptides in two reverse titrations, first titrating Ni-ZF with Co(II) followed by the titration of Co-ZF with Zn(II), allows one to obtain very convergent data

when compared with the results obtained via the competition and potentiometry techniques. Using the different techniques, the pK_d value of the Zn-ZF133-11 complex was determined to be 12.55 (chelator competition), 12.42 (potentiometry), and 12.48 (modified three-step titration method) with an average value of 12.4 ± 0.1.

Limitations of the experimental methods

Our results demonstrate that application of the widely used spectrophotometric approach for the determination of the Zn(II) affinity of proteins (in this case, to zinc fingers) leads to the overestimation of the dissociation constants (and an underestimation of the affinity) if the stability of the complex is high. In a fact, an overestimation is commonly observed if the K_d is lower than 10^{-7} M; however, it also depends on the particular zinc finger peptide, its concentration, the equilibration time and many other factors. Although this fact was recently observed by Sénèque and Latour for non-natural zinc fingers, such as CP1 (consensus zinc finger peptide), which has various coordination modes, and its truncation, the cyclic L_{TC} and L_{HSP} peptides.[13] They demonstrated that the dissociation constants of the model zinc finger peptides determined during competition with chelators are between two and four orders of magnitude lower than those previously determined spectrophotemtrically.[13] The detailed approach of the spectrophotometric titration method presented here demonstrates that processing of the experimental data of a tightly binding metal-ligand system results in a significant deviation from the correct values. A meaningful value might be determined only when the limiting species are distributed between the two forms (first: the peptide bound with the metal ion, second: unbound), with the respective fraction of the first form below 0.95 (ideally below 0.9).[36-38] Therefore, sharp titration endpoints are the major limiting factor in the precise determination of high stability constants and clearly demonstrate the lack of or limited effective equilibrium competition between species. It is useful to obtain information

regarding the complex stoichiometry rather than an accurate stability.[20] One method to avoid an overestimation of the dissociation constant is to decrease the fraction bound to the metal species by lowering the concentration of reactants (Figure 7); however, this is frequently limited by the sensitivity of the instrument and leads to an increased experimental error. The values presented in Table 1 confirm the above statement. The K_d^* values of the Co-ZF complexes determined at a concentration of 3.5 μM are lower and closer to accurate values (K_d). The simulations of the spectrophotometric titration curves in Figure 7 confirm our observation regarding the different K_d^* values determined at the different zinc finger concentrations. Because the titration of zinc finger peptides with Co(II) at concentrations lower than the μM scale is impossible (due to low extinction coefficients and instrument sensitivity), we can expect that zinc fingers with K_d values below 10^{-7} M are determined via direct titrations and have an error that increases proportionally the lower the K_d value (Figure 7c). Even a small change in the titrated species, such as the uniqueness of the instrument, oxidation, heterogeneity of the sample, etc., may result in a major difference in the fitted dissociation constant from one experiment to the next. The titration of Ni-ZF with Co(II) and Co-ZF with Zn(II), as was demonstrated above, results in different pK_d^* values if the competing metal ion is not in great enough excess. Increasing the metal ion concentration results in metal hydroxide precipitation, and the concentrations of the metal ions used in this experiment were at the maximum permissible levels. Figure 6b shows that an excess of the total amount of Ni(II) in the reverse-titration results in a clearly visible change in the shape of the titration curve, which proves the accuracy of our results. Re-evaluation of previously published data for the MTF1-1 and Sp1-3 zinc fingers demonstrates that use of the K_d values of the Co-ZF complexes determined in this study automatically change the Zn-ZF dissociation constants to a value that is much closer to those determined in the chelator competition. Figure 6c indicates however, that the difference between the peptide saturation with Zn(II) at

different Co(II) concentrations does not significantly affect the shape of the titration curve (inset of Figure 2c). However, the K_d* values determined at different Co(II) concentrations vary. Again, a re-evaluation of the previous results will likely match those obtained in this study. Therefore, the limitation of reverse-titration is the high Zn(II) to protein affinity. If any zinc finger binds to the Zn(II) with a K_d lower than 10^{-13} M, the application of this method will likely result in, once again, the overestimation of the K_d. The comparison of the data obtained with the different techniques is therefore critical for an accurate evaluate of the metal to peptide affinity.

The other techniques presented here to determine the high Zn(II) to protein affinities also have limitations. The use of circular dichroism (CD) for monitoring zinc finger saturation is limited, due to conformational changes of the peptide. If the ellipticity of the zinc finger does not change significantly upon Zn(II) binding, the applicability of the method is also limited. Moreover, the presence of chelating agents as well as absorbing buffer components limit the use of CD to the near-UV range, where the changes are normally minor. The accuracy in the determination of the affinity with metal buffers requires the change in the metal/competitor ratio upon metal ion transfer from the buffer to the apo-form of zinc finger to be taken into account. Determination of the dissociation constant in the presence of a competitor requires knowledge of the accurate stability constants of the competitor to the metal ion. The application of potentiometry was discussed above, and its limited use statistically depends on the type of zinc binding motif. A high number of amino acid residues with acid-base properties prevents the proper and quantitative determination of all of the equilibria.

Variations in the stability of the zinc finger metallome

The knowledge of Zn(II) affinity for proteins is a critical issue in the understanding of the function of macromolecules and the role of Zn(II) in the modulation of their activity. Intracellular zinc proteins bind Zn(II) very tightly, with picomolar and femtomolar affinities.[1] The dissociation constants of zinc enzymes normally vary from 10^{-11} to 10^{-12} M, although lower Zn(II) affinities have also been found in the literature.[1, 2] The reported zinc finger dissociation constants vary significantly from submicromolar to attomolar and do not fully cover the cellular Zn(II) availability.[7-13] How this diversity is achieved with the same or similar coordination sphere is still unanswered.[39] Thus far, we know that Zn(II) affinity is primarily controlled by the second coordination sphere and the number of stabilizing intramolecular interactions.[19, 40, 41] Hydrophobic, as well as electrostatic interactions, with a number of hydrogen bonds stabilize both the thermodynamic and kinetic stability.[13] Despite these stabilizing effects, it is still unclear how and whether the stability constants of ββα zinc fingers determined on the same peptide type differ by so many orders of magnitude.[16, 17] The conditions and methodology used for estimating the constants are likely factors affecting their values. In a recent article by Sénèque and Latour on zinc finger stability, it was shown that Zn-ZF and Co-ZF complexes have higher stability than previously reported.[13] The reason why different methods provide different results still remains unresolved. It should be noted that this observation is based on the use of non-natural zinc fingers, including cyclic peptides, which have a restrained conformation. Studies performed on a prototype of zinc finger consensus CP1 peptides, showed that their dissociation constants are as low as 10^{-14}-10^{-15} M. The consensus zinc finger introduced by Berg and co-workers, based on 131 ββα zinc finger sequences, has a number of conserved amino acid residues that stabilize its structure and thus possess high Zn(II) affinity. Therefore, CP1 is not a relevant stability model for natural zinc fingers.[31] Here, we focused on natural ββα zinc fingers that do not possess a number of

stabilizing effects that are included in CP1. Studies performed on natural zinc fingers, using three independent methods, showed that the Zn-ZF dissociation constants are clearly higher than the values obtained for CP1 and lower than previously reported for various natural zinc fingers. The above results demonstrate that use of the common reverse-titration method for the determination of dissociation constants has major limitations, which in fact leads to the overestimation of K_d values. As an alternative, we present a modified reverse-titration method, whose application provides results that are convergent with the other common methods. Because the dissociation constants determined here for natural zinc fingers are compact and vary between ~10^{-11} and 10^{-13} M, it is still not fully understood why the values for CP1 are significantly lower (~10^{-16} M). Figure 2c compares the pK_d values of Zn-MTF1-1, Zn-Sp1-3, Zn-ZF133-11, Zn-ZF278-1, and Zn-CP1 with the average pK_a values determined in the pH titrations (Figure 2b). Both values for the CP1 zinc finger were taken from the literature and correspond to a pH of 7.4.[13] Surprisingly, the thermodynamic data for all of the discussed zinc fingers are linearly correlated (R^2 = 0.99), which confirm their accuracy based on the type of determination. This correlation underlines and, to some extent, explains the difference between natural and consensus ββα zinc fingers. Although natural zinc fingers have many major similarities (Figure 1), they still differ substantially, for example, in the number of amino acid residues present between the coordinating cysteines or the number of polar and hydrophobic residues. A difference of nearly five orders of magnitude in the dissociation constants (Figure 2c) of the highly conserved ββα zinc fingers reveals that huge energetic effects must be hidden in the short amino acid sequence and 3D structure of the domain. It has been shown that conformational rearrangements of zinc fingers contribute to the stability of the entire complex and the complexity is entropically driven due to the dissociation of the thiol protons at pH 7.4.[42-44] However, the entropy and enthalpy components have been shown to be equal in Zn-Sp1-3, which demonstrates an enthalpy-entropy compensation process

found in ββα zinc fingers.[22] The other residues also contribute to the stability of these domains. Solvation enthalpy has also been shown as an example that contributes to protein stability.[45]

Our findings show that the stability of natural ββα zinc fingers is not as high as recently reported for CP1, and the two types differ from one another. This is in contrast to the common statement that zinc fingers are only structural zinc sites that are always occupied. Our results however, focusing in part on the determination of zinc finger stability, show that some naturally occurring zinc fingers may potentially remain unoccupied and are transiently loaded with Zn(II) in the cell. However, this statement must be confirmed using a multitude of different zinc fingers *in vivo*. Large variations in the stability constants within the same class of zinc fingers reveal that hidden thermodynamic effects in the zinc finger sequences are the key elements responsible for the differentiation of the stability of the zinc finger metallome.

Conclusions

In this study, we demonstrated that the affinity of Zn(II) for zinc fingers differs when compared with the results of commonly employed reverse-titration methods. There are limitations when processing the spectrophotometric titration data, with regards to various different zinc finger peptides and the concentrations of competing metal ions. To improve the robustness of the spectrophotometric method, we demonstrated that the additional titration of the zinc fingers with Ni(II) prevents the overestimation of the Co(II) and Zn(II) dissociation constants. We also discussed the usage and limitations of the other experimental methods, such as potentiometry and competition with metal chelators. Finally, we showed that natural zinc fingers are much less stable than those based on consensus sequences due to a number of stabilizing effects found in the latter. These findings have important biological implications regarding zinc finger functionality under the physiological availability of Zn(II). The fluxes of

intracellular free Zn(II) concentrations, which have been shown to be within the range of 10^{-9} to 10^{-11} M, may impact the saturation and protein function of certain zinc fingers. The variation in zinc finger stability within the same, highly conserved class of zinc fingers shows that the stabilization effects hidden both in the sequence and structure of particular zinc fingers are crucial for the differentiation of the stability and functionality of the zinc finger metallome.

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Table 1. Comparison of the dissociation constants (pK_d^* and pK_d)[a] of the Ni(II), Co(II) and Zn(II) complexes formed with the zinc finger peptides, as determined by various methods. The pK_a value refers to an average dissociation constant of the amino acid residues bound to Zn(II). The numbers in brackets refer to the standard errors of the last digit.

Zinc finger peptide	pK_d^*				pK_d					pK_a
	Co(II)	Co(II)	Zn(II)	Zn(II)	Ni(II)	Co(II)	Zn(II)	Zn(II)	Zn(II)	pH titration
	Directly (35 μM ZF)	Directly (3.5 μM ZF)	Reverse-titration	Ref.	Directly	2.5 mM Ni(II)	3.5 mM Co(II)	Metal buffers	Potentio-metry	
MTF1-1	6.70 (6)	7.4 (2)	9.7 (1)	10.5[b]	4.68 (4)	7.24 (3)	11.44 (8)	11.62 (3)	-	4.80 (1)
Sp1-3	6.00 (2)	5.85 (4)	8.3 (1)	8.5[c], 9.22[d]	5.53 (3)	8.64 (4)	12.72 (7)	12.70 (1)	-	4.57 (1)
ZF133-11	6.61 (5)	7.4 (2)	9.66 (8)	-	5.50 (4)	8.44 (4)	12.42 (3)	12.55 (3)	12.48	4.65 (2)
ZF278-1	7.33 (9)	8.4 (4)	10.2 (2)	-	5.46 (3)	8.72 (3)	12.89 (6)	13.04 (2)	-	4.55 (1)

[a] indicates differences in the constant values obtained by different methods, K_d^* refers to a value that is not comparable with the accurate K_d value.
[b] reference 18.
[c] reference 16.
[d] reference 17.

Table 2. The cumulative protonation and stability constants (log β^{a}) of the ZF133-11 peptide and its Co(II) and Zn(II) complexes. The numbers in brackets denote the standard deviations of the last digit.

Species	log β		
	ZF133-11 (L)	Co(II)	Zn(II)
HL^{3-}	9.62 (1)		
H_2L^{2-}	18.25 (1)		
H_3L^{-}	25.86 (2)		
H_4L	32.18 (2)		
H_5L^{+}	38.08 (2)		
H_6L^{2+}	42.48 (2)		
H_7L^{3+}	46.40 (2)		
MH_3L^{+}		29.0 (2)	33.27 (5)
MHL^{-}		19.61 (4)	23.73 (5)
ML^{2-}		9.64 (9)	13.60 (9)
$MH_{-1}L^{3-}$		-0.32 (6)	3.17 (6)

[a] $\beta\ M_iH_jL_k = [M_iH_jL_k] / ([M^{2+}]^i\ [H^+]^j\ [L^{4-}]^k)$, where L^{4-} is the fully deprotonated anion of ZF133-11.

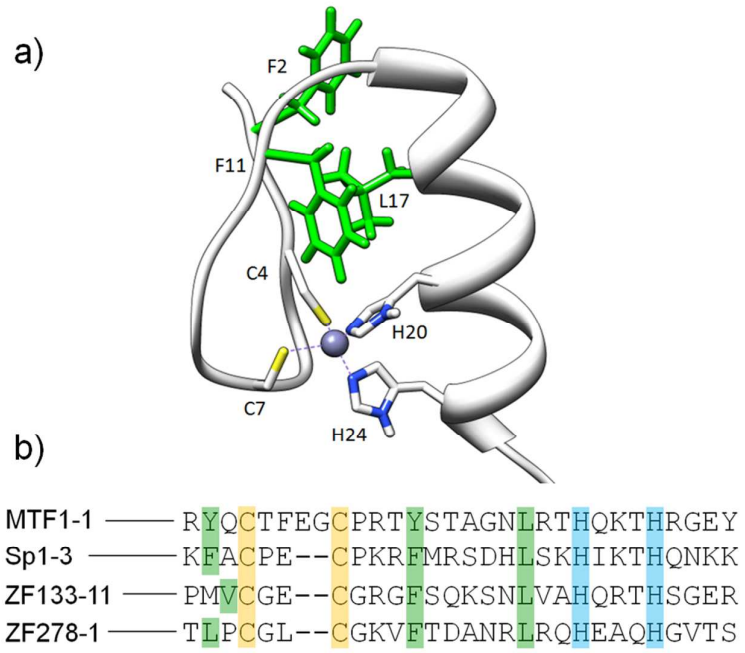


Figure 1. The 3D structure and sequences of the ββα zinc fingers used in this study. a) NMR structure of the Sp1-3 zinc finger based on the pdb 1va3 deposition. b) The amino acid sequences of the zinc finger peptides used in this study. The green color denotes residues that are responsible for the formation of the hydrophobic interactions in most classical zinc fingers (F2, F11 and L17 for Sp1-3). The yellow and blue indicate the cysteine and histidine residues, respectively, responsible for the Zn(II) binding.

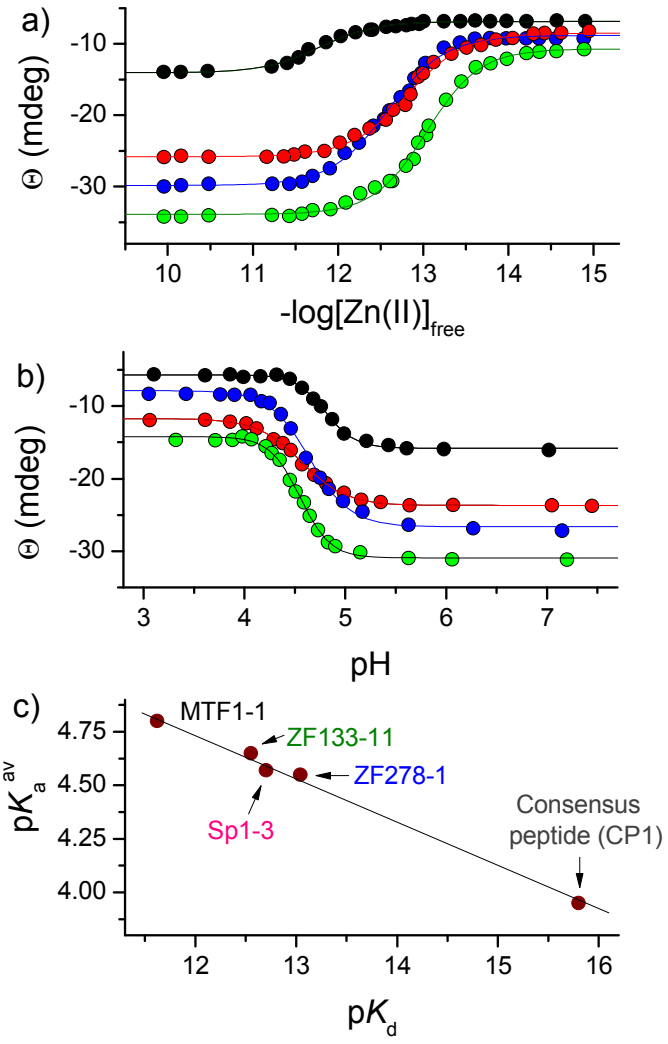


Figure 2. The binding of Zn(II) to the zinc finger peptides, determined spectropolarimetrically. The black, red, green and blue colors correspond to the MTF1-1, Sp1-3, ZF133-11 and ZF278-1 zinc fingers, respectively. (a) Isotherms of the zinc fingers saturated with Zn(II) in a set of metal buffers at pH 7.4. (b) Determination of the pK_a^{av} values of the zinc finger complexes. (c) Linear correlation of the determined pK_d and pK_a^{av} values. The values for the CP1 peptide were taken from reference 13.

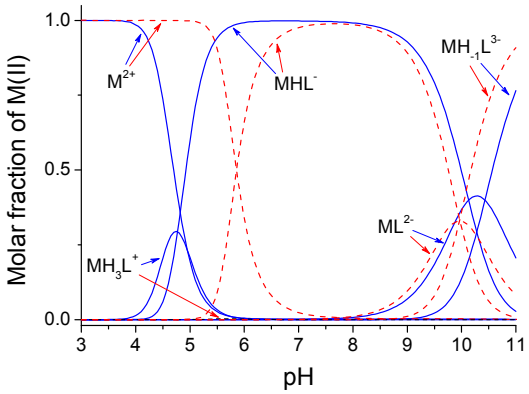


Figure 3. Molar fraction distribution of the Zn(II) (blue line) and Co(II) (red dashed line) complexes with ZF133-11 in a wide range of pH. The graph was prepared for 50 μM of Zn(II)-ZF133-11 based on the log β constant values from Table 2.

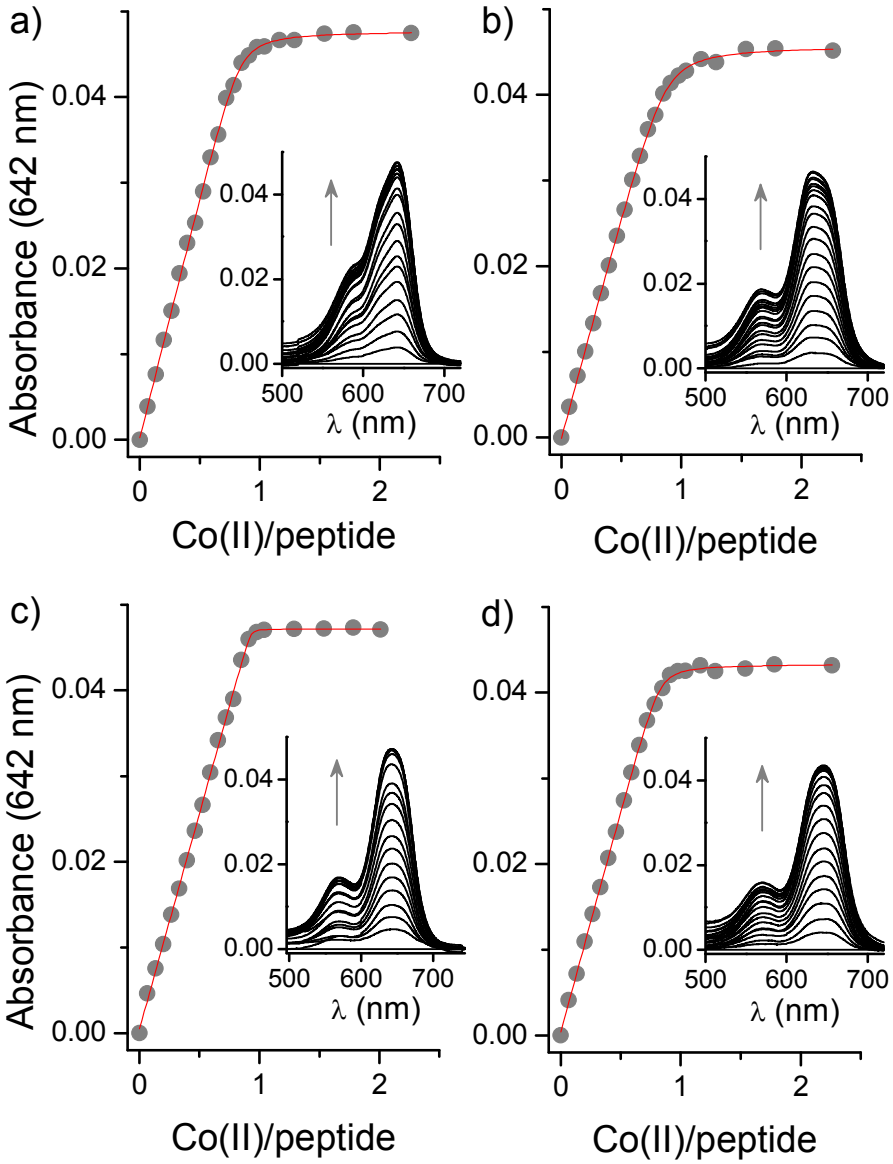


Figure 4. Spectrophotometric titrations of 50 μM of the MTF1-1 (a), Sp1-3 (b), ZF133-11 (c) and ZF278-1; (d) the zinc finger peptides with Co(II) in 50 mM HEPES, pH 7.4 (I = 0.1 from $NaClO_4$), at 25°C. TCEP was added to a final concentration of 250 μM. The experimental points collected at 642 nm were fitted with Equation 3 using Origin 8.1 software.

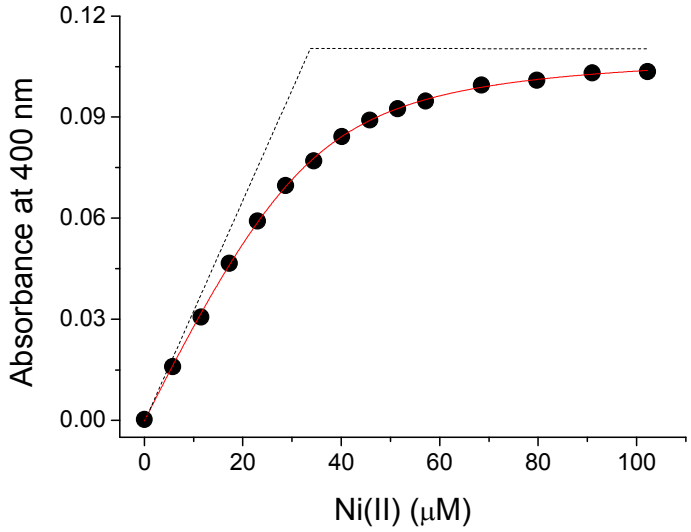


Figure 5. Titration of 35 μM of ZF133-11 with Ni(II) in 50 mM HEPES buffer, pH 7.4 (I = 0.1 from $NaClO_4$), monitored at 400 nm. TCEP was added to a final concentration of 250 μM. The experimental points were fitted to Equation 3 using Origin 8.1 software. The dissociation constant of the Ni(II)-ZF133-11 complex is 4.54 μM. The dashed line demonstrates theoretical titration curve typical for the high affinities (K_d below 10^{-7} M).

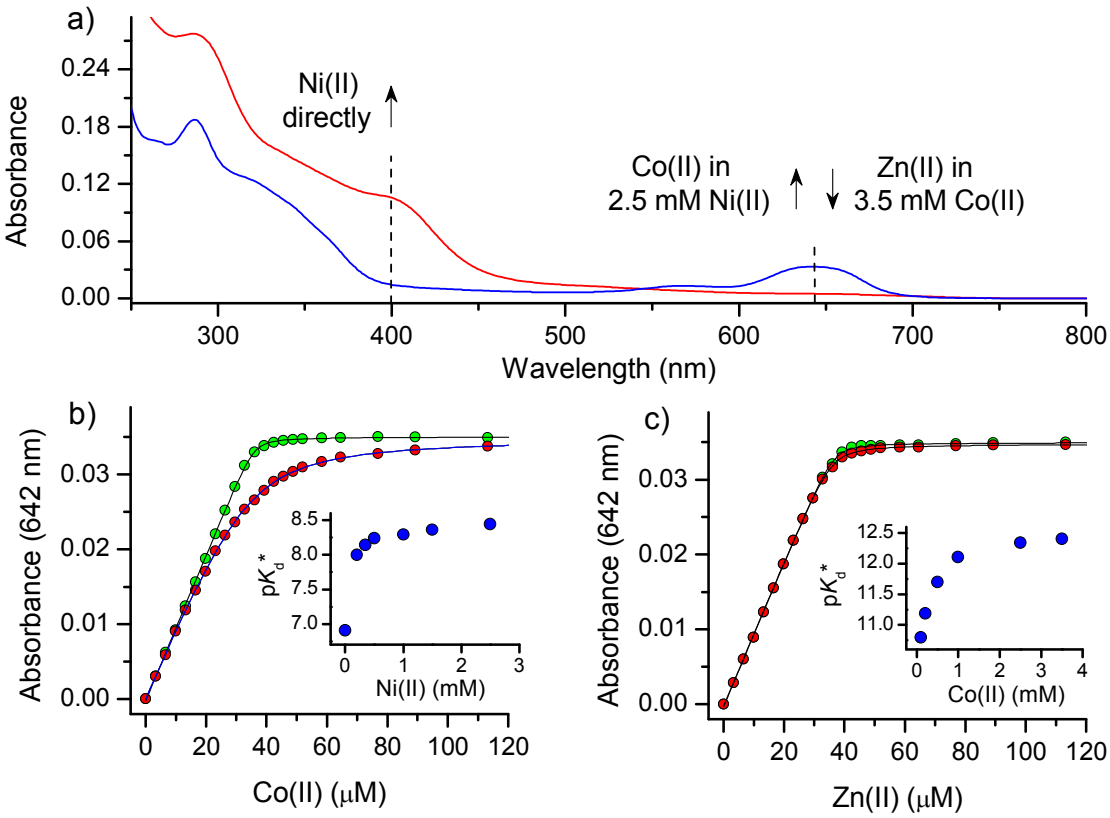


Figure 6. Illustration of the three-step titration method developed in this study for the determination of the Zn(II)-ZF dissociation constants. (a) UV-Vis spectra of the Ni(II) (red) and Co(II) (blue) complexes of ZF133-11. The dashed line and arrows indicate the chosen wavelengths for the spectroscopic titrations and the absorbance increase or decrease tendency. (b) Comparison of the titrations of ZF133-11 in the presence of 0 (green) and 2.5 mM (red) of Ni(II) with Co(II). (c) Comparison of the reverse titrations of the Co(II)-ZF133-11 complex in the presence of 0.1 (green) and 3.5 mM (red) of Co(II) with Zn(II). The decreasing absorbance at 642 nm was transformed proportionally to the increasing values. The insets in graphs (b) and (c) demonstrate the changes in the pK_d^* values determined at increasing concentrations of competing Ni(II) and Co(II), respectively.

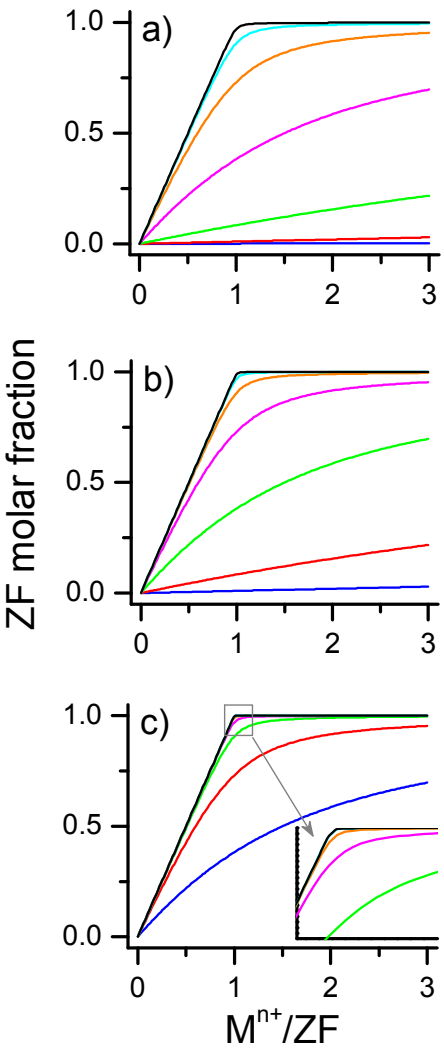
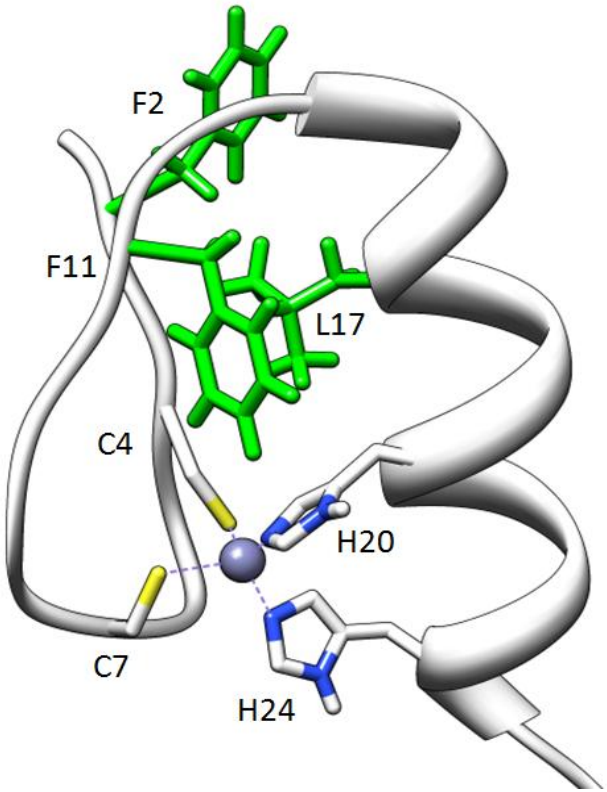


Figure 7. Simulation of the spectrophotometric titrations of the zinc finger peptide with a metal ion (M^{n+}) at different peptide concentrations: (a) 100 nM, (b) 1 μM, (c) 100 μM. The dark blue, red, green, magenta, orange, light blue and black colored lines represent the pK_d values from 4 to 11, respectively. The inset in (c) is a magnification of the end-points of the titrations.

a)



b)

```
MTF1-1   ——— RYQCTFEGCPRTYSTAGNLRTHQKTHRGEY
Sp1-3    ——— KFACPE--CPKRFMRSDHLSKHIKTHQNKK
ZF133-11 ——— PMVCGE--CGRGFSQKSNLVAHQRTHSGER
ZF278-1  ——— TLPCGL--CGKVFTDANRLRQHEAQHGVTS
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