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Evaluation of the Intrinsic Zn(II) Affinity of a Cys_3His_1 Site in the Absence of Protein Folding Effects

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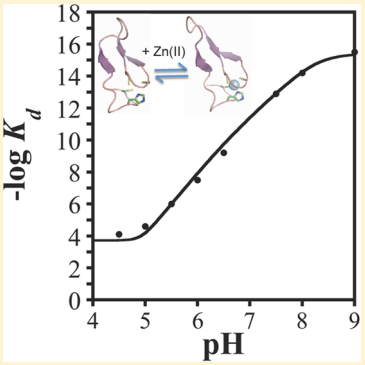
Supporting Information

ABSTRACT: Zinc finger transcription factors are the largest class of metalloproteins in the human genome. Binding of Zn(II) to their canonical Cys_2His_2, Cys_3His_1, or Cys_4 sites results in metal-induced protein folding events required to achieve their biologically active structures. However, the coupled nature of metal binding and protein folding obscures the individual free energy contributions of each process toward overall zinc finger stabilization. Herein, we separate the energetic contributions of metal−ligand interactions from those of protein−protein interactions using a natural protein scaffold that retains essentially identical structures with and without Zn(II) bound, the 59 amino acid zinc binding domain of human transcription factor IIB (ZBD-TFIIB). The formation constant of Zn(II)-ZBD-TFIIB, which contains a single Cys_3His_1 site, was determined to be 1.5×10^{15} M^{-1} via fluorimetry and isothermal titration calorimetry. Isothermal titration calorimetry showed that Zn(II) binding is entropically favored at pH 5.5, 7.0, and 8.0 and enthalpically favored at pH 8.0 but slightly enthalpically disfavored at pH 5.5 and 7.0. The conditional dissociation constants of Zn(II)-ZBD-TFIIB and natural Cys_3His_1 zinc finger proteins were compared to determine the free energy cost of protein folding in the latter. Our analysis reveals that the energetic cost to fold zinc finger proteins is minimal relative to the contribution of Zn(II) binding and suggests that the true role of Zn(II) binding may be to modulate protein dynamics and/or kinetically template the protein folding process.

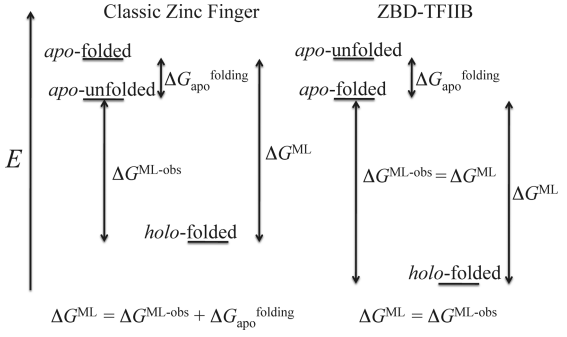


INTRODUCTION

The largest class of metalloproteins encoded in the human genome,[1] the zinc finger transcription factor proteins,[2,3] exhibits metal-induced protein folding events.[4,5] The incorporation of Zn(II) into their pseudotetrahedral Cys_2His_2, Cys_3His_1, or Cys_4 sites results in the formation of their folded, active *holo*-state from an unfolded ensemble. The coupled thermodynamics of metal-ion binding and protein folding serve to obscure the individual free energy contributions of each toward metalloprotein conformational stability.[6] Despite this fundamental ambiguity, the current dogma is that the role of Zn(II) binding is to thermodynamically drive protein folding by as much as +16 kcal/mol.[7]

Metal-ion binding constants[8,9] are sensitive probes for relaying thermodynamic information about protein structural changes. Indeed, the Co(II) binding constants of a synthetic zinc finger peptide, Consensus Peptide 1 (CP1), were used to develop an amino acid β-sheet propensity scale.[10] Scheme 1 shows that, for a classic zinc finger protein with a metal-induced protein folding event, the actual free energy contribution of metal-ion binding, ΔG^{ML}, differs from the observed contribution, $\Delta G^{ML\text{-}obs}$, by the unfavorable free energy contribution of protein folding, $\Delta G_{apo}^{folding}$. However, in the absence of a metal-induced protein folding event, the observed and actual free energy contributions of metal binding are equivalent, $\Delta G^{ML\text{-}obs} = \Delta G^{ML}$. Thus, a comparison of the metal-ion binding constants from Zn(II) proteins with and without metal-induced protein folding events allows for a determination of the free energy cost of protein folding in the former.

Scheme 1. Zinc Protein Folding Thermodynamics



Our previous work using a model peptide scaffold suggested that the cost of protein folding in zinc finger proteins was

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minimal compared to the free energy contribution of Zn(II) binding.[11−13] This conclusion was based on a comparison of the free energy of Zn(II) binding between a simple, unstructured 16 amino acid peptide, **GGG**, with an assumed zero kcal/mol cost of protein folding and natural zinc finger peptides with the same coordination motif.[13] Using this method, we[14] and others[15,16] find that the cost of protein folding in most natural zinc fingers is minimal, $\Delta G_{apo}^{folding} \leq$ +4.2 kcal/mol, compared to the approximately −15 kcal/mol favorable free energy contribution of Zn(II) binding at physiological pH.

Herein we use metal-ion binding constants to determine ΔG^{ML} for Zn(II) binding to a Cys_3His_1 site in a protein without any protein folding effects to determine the cost of protein folding in natural Cys_3His_1 zinc fingers. Figure 1 shows that the

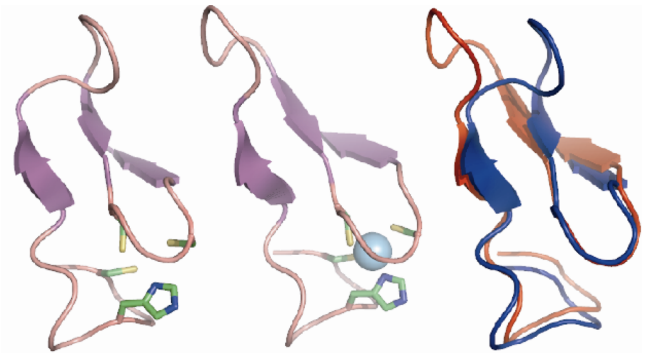


Figure 1. Solution NMR structures of human transcription initiation factor IIB in the *apo*-state (PDBID 1RO4[17]) and the *holo*-state (PDBID 1RLY[17]), and the two structures superimposed.

apo- and *holo*-structures of the zinc binding domain of human transcription initiation factor IIB (ZBD-TFIIB) are essentially identical and there are no protein folding effects associated with Zn(II) binding.[17] The inherent pH-independent formation constant for Zn(II)-ZBD-TFIIB is determined using equilibrium thermodynamic measurements of Zn(II) affinity over the pH range of 4.5−9.0 followed by fluorescence spectroscopy and isothermal titration calorimetry. These Zn(II)-ZBD-TFIIB data are compared to natural zinc finger proteins to reveal the cost of protein folding in the latter.

EXPERIMENTAL SECTION

Materials. Zinc(II) chloride, trifluoroacetic acid, and isopropyl β-D-1-thiogalactapyranoside (IPTG) were obtained from the Sigma-Aldrich Chemical Co. Aqueous stock solutions of Zn(II) were quantified by atomic absorption spectroscopy. All other chemicals and solvents were reagent grade and used without further purification.

Expression and Purification of ZBD-TFIIB. The pET3a plasmid containing the gene encoding the 59 amino acid zinc binding domain of human transcription factor IIB was generously provided by Prof. Mitsuhiko Ikura (University of Toronto). The plasmid was transformed into *Escherichia coli* strain BL21(DE3) by electroporation. Cultures were grown at 37 °C in Luria-Burtani broth containing 200 μg/mL of ampicillin. When cultures reached an optical density of 0.8−0.9 at 600 nm, ZBD-TFIIB synthesis was induced by addition of isopropyl β-D-1-thiogalactapyranoside (IPTG) to 0.5 mM and allowed to grow an additional 3 h.

Cell paste from a 2 L culture was resuspended in 50 mL of lysis buffer (20 mM Tris-HCl, pH 7.5, 25 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 10 μg/mL pepstatin, 10 μg/mL leupeptin, and 2 mM EDTA). The suspended cell paste was then ruptured by application of 11 000 PSI of pressure in a French Press. The insoluble and soluble fractions of the cell lysate were separated by centrifugation at 27 000*g* for 30 min at 4 °C. The soluble fraction of the lysate was dialyzed against 0.1% (v/v) trifluoroacetic acid in water.

The crude lysate containing the desired ZBD-TFIIB protein was purified to homogeneity by reversed phase C_{18} HPLC using aqueous−acetonitrile gradients containing 0.1% (v/v) trifluoroacetic acid. After the collected fractions were lyophilized, the purity was determined by analytical HPLC (>95% purity), and the identity of the resulting protein was confirmed with matrix-assisted laser desorption mass spectrometry: expected m/z = 6433.2, observed m/z = 6433.0. The sequence of ZBD-TFIIB is as follows with the metal ligands in bold: NH_2-ASTSRLDALPRVT**C**PN**H**PDAILVEDYRAGDMI**C**P-E**C**GLVVGDRVIDVGSEWRTFSNDKA-COOH

UV−Vis Spectroscopy. UV−vis spectra were recorded on either a Varian Cary 100 or a Cary 300 spectrophotometer using anaerobic quartz cells of 1.0 cm path length. Peptide concentrations were determined spectrophotometrically using ε_{280} of 7000 M^{-1} cm^{-1} for ZBD-TFIIB.

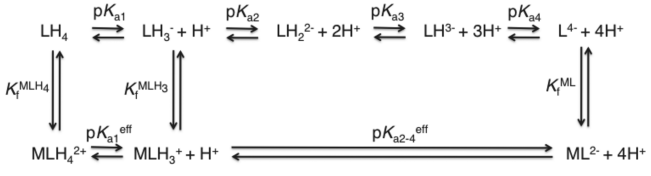
Fluorescence Spectroscopy. Excitation and emission fluorescence spectra were recorded on a Cary Eclipse fluorimeter using anaerobic quartz cells of 1.0 cm path length. Excitation and emission slit widths of 5.0 and 2.5 nm were employed. The excitation wavelength was 280 nm, and the fluorescence emission was collected from 300 to 450 nm. Peptide concentrations were between 1 and 25 μM as determined spectrophotometrically using ε_{280} = 7000 M^{-1} cm^{-1} for ZBD-TFIIB.

Isothermal Titration Fluorimetry. Direct Zn(II) Titrations. Aqueous stock solutions of $Zn(II)Cl_2$ were added in microliter aliquots to freshly prepared peptide solutions in aqueous buffers (20 mM MES, 100 mM KCl) under strictly anaerobic conditions in 1.0 cm cuvettes. Samples were allowed to equilibrate for 5 min before measurement of their fluorescence spectra. The conditional metal−ligand dissociation constants, conditional K_d values, were obtained from fitting a plot of the increase in tryptophan fluorescence at 355 nm against the [Zn(II)]/[peptide] ratio to a 1:1 equilibrium binding model.

Isothermal Titration Fluorimetry. EDTA Competition Titrations. At pH values above 6.0, conditional equilibrium dissociation constant determinations for Zn(II)-ZBD-TFIIB necessitated ethylenediaminetetraacetic acid, EDTA, competition. To buffered aqueous solutions (20 mM HEPES, 100 mM KCl) of 10−15 μM peptide and 1.0 equiv of Zn(II), EDTA was titrated in microliter aliquots under strictly anaerobic conditions. The decrease in fluorescence at 355 nm upon the addition of EDTA is fit to a 1:1 competition model. The K_{comp} value, coupled with the conditional equilibrium dissociation constant value of Zn(II)-EDTA, $K_d^{Zn(II)\text{-}EDTA}$, gives the conditional equilibrium dissociation constant value for Zn(II)-ZBD-TFIIB.

pH Dependence of Conditional Dissociation Constants. Due to the expected proton dependence of the Zn(II)-ZBD-TFIIB conditional dissociation constants, K_d values were measured at varying pH values in order to determine the value of K_f^{ML}. The K_d values of Zn(II)-ZBD-TFIIB at each pH were determined as above using fluorescence spectroscopy. Since the observed K_d values of Zn(II)-ZBD-TFIIB and **GGG**-Cys_3His_1 are identical, within the 1.0 kcal/mol error, the Zn(II)-ZBD-TFIIB data were fit to the same equilibrium model developed for **GGG**-Cys_3His_1 shown in Scheme 2.[13] The plot of −log K_d versus pH was fit to the equilibrium binding expression for the pH dependent formation of Zn(II)-ZBD-TFIIB from the ZBD-ZBD-TFIIB protein and $\{Zn(II)(H_2O)_6\}^{2+}$, as shown in eq 1.

Scheme 2. Equilibrium Model for Zn(II)-ZBD-TFIIB Formation



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$$-\log K_d = -\log\left(\frac{1}{K_f^{ML}} * \frac{1+10^{(-pH+pK_{a1}^{eff})}+10^{(-4pH+pK_{a1}^{eff}+3pK_{a2\text{-}4}^{eff})}}{1+10^{(-pH+pK_{a1})}+10^{(-2pH+pK_{a1}+pK_{a2})}+10^{(-3pH+pK_{a1}+pK_{a2}+pK_{a3})}+10^{(-4pH+pK_{a1}+pK_{a2}+pK_{a3}+pK_{a4})}}\right) \quad (1)$$

The conditional dissociation constant at any pH, K_d, is a function of the pH-independent formation constant, K_f^{ML}; the effective acid dissociation constants of the metal-bound histidine, pK_{a1}^{eff}, and cysteines, pK_{a2-4}^{eff}; the acid dissociation constant values for the histidine, pK_{a1}, and cysteines, pK_{a2}, pK_{a3}, and pK_{a4}, in the *apo*-protein; and the solution pH.

Isothermal Titration Calorimetry (ITC). All ITC measurements were performed on a MicroCal VPN-200 titration calorimeter. All manipulations were performed under strictly anaerobic conditions with buffers degassed using successive freeze−pump−thaw cycles in a glovebox. An aqueous stock solution of 2.0 mM $Zn(II)Cl_2$ was added into aqueous buffers containing 15−50 μM freshly prepared protein. Below pH 9.0, $Zn(II)Cl_2$ in the solution is predominantly $\{Zn(H_2O)_6\}^{2+}$. To mitigate for the spurious and unaccounted heats produced by the addition of the stock zinc solution, whose identity is not identical to the buffered protein solution, the heat of addition of the metal solution was determined in each ITC experiment and subtracted from the ITC data. These heats were comparable to the heats of addition of the zinc stock solution to the buffers without the protein.

Titrations were performed in triplicate using three different buffers at each of three pH values, 5.5, 7.0, and 8.0. At all pH values, 20 mM HEPES, 100 mM KCl ($\Delta H_{protonation}$ = −5.02 kcal/mol),[18] and 20 mM PIPES, 100 KCl ($\Delta H_{protonation}$ = −2.73 kcal/mol),[18] were used. The third buffer used for pH 8.0 was 20 mM MOPS, 100 mM KCl ($\Delta H_{protonation}$ = −5.22 kcal/mol),[18] whereas 20 mM MES, 100 mM KCl ($\Delta H_{protonation}$ = −3.71 kcal/mol),[18] was used as the third buffer for pH 5.5 and 7.0. The pH of the sample was measured before and after the experiment to ensure that no change in pH occurred. Between experiments, the sample cell was washed with 0.5 mM EDTA and thoroughly rinsed with deionized water to remove residual protein, metal-ions, and EDTA.

The Origin software supplied with the MicroCal instrument was used to analyze all the data. The reaction enthalpies (ΔH_{rxn}'s) were determined from the observed enthalpy (ΔH_{obs}) and the buffer protonation enthalpy ($\Delta H_{bufferprotonation}$) scaled by the number of protons released (n) during the reaction, determined by measuring the slope of ΔH_{obs} versus $\Delta H_{buffer\ protonation}$ across three buffers, and the solution pH value using the following equation:

$$\Delta H_{obs} = \Delta H_{rxn} + n\Delta H_{bufferprotonation} \quad (2)$$

The number of protons released for Zn(II)-ZBD-TFIIB at each pH value was found to be equivalent to that determined for Zn(II)-**GGG**-Cys_3His_1 from the intrinsic and effective pK_a values of the Cys and His ligands, supporting our use of these Zn(II)-**GGG**-Cys_3His_1 values in the fit of the pH dependence of the conditional dissociation constants for Zn(II)-ZBD-TFIIB. The reaction entropies were calculated on the basis of the calorimetric determination of the reaction enthalpies, and the reaction free energies (from ITC or fluorescence titrations), according to the following relationship:

$$\Delta G_{rxn} = \Delta H_{rxn} - T\Delta S_{rxn} \quad (3)$$

RESULTS

Experimental Design. Natural proteins often use the favorable free energy contribution of metal-ion coordination to effect protein conformational change.[19,20] Zinc finger proteins are among the most well-known[6−8] and widespread[21] biological macromolecules that undergo metal-induced protein folding events. Lacking well-defined secondary structure in the *apo*-state, zinc fingers fold into discrete three-dimensional structures upon Zn(II) complex formation. Scheme 1 shows a free energy diagram of the coupled metal-induced protein folding event of a classic zinc finger protein. The free energy required to fold the *apo*-protein into the *apo*-folded state, i.e., the structure observed in the *holo*-protein but without the metal bound, is given by $\Delta G_{apo}^{folding}$. The conditional dissociation constant, K_d value, measured for the transition from the ensemble of *apo*-unfolded states to the *holo*-folded state upon metal-ion binding, can be used to determine the observed metal−ligand binding free energy, $\Delta G^{ML\text{-}Obs}$, via the relationship $\Delta G^{ML\text{-}Obs} = -RT \ln K_d$. However, the *observed* free energy of metal binding, $\Delta G^{ML\text{-}Obs}$, is less than the *actual* metal−ligand free energy contribution, ΔG^{ML}, by the cost of protein folding, $\Delta G_{apo}^{folding}$, i.e., $\Delta G^{ML} = \Delta G^{ML\text{-}Obs} - \Delta G_{apo}^{folding}$. The inability to directly measure ΔG^{ML} or $\Delta G_{apo}^{folding}$ has made it difficult to ascertain how much metal coordination free energy actually drives the protein folding event, and literature estimates for $\Delta G_{apo}^{folding}$ range from 0 to +16 kcal/mol.[7]

We developed a method to use the conditional binding constants from a designed peptide to determine the cost of protein folding in natural zinc finger peptides and proteins.[13] Our method is based on a simple, unstructured 16 amino acid peptide, **GGG**, into which we individually placed each of the canonical zinc finger metal-ion coordination motifs, Cys_2His_2, Cys_3His_1, and Cys_4. The resulting peptides, **GGG**-Cys_2His_2, **GGG**-Cys_3His_1, and **GGG**-Cys_4, each bind Zn(II) in a 1:1 stoichiometry and have formation constant values of 2.5×10^{13} M^{-1}, 1.5×10^{15} M^{-1}, and 5.6×10^{16} M^{-1}, respectively.[13] Since the **GGG** peptides have no secondary structure in the *apo*- and *holo*-forms, their free energy cost of protein folding is assumed to be minimal, $\Delta G_{apo}^{folding} = 0$ kcal/mol. Thus, it was assumed that $\Delta G^{ML\text{-}obs} = \Delta G^{ML}$ for the **GGG** peptides and that the difference in the Zn(II) affinity between a natural or designed zinc finger peptide (ZFP) and the **GGG** peptide with the same coordination motif could be delineated using eq 4.

$$\Delta G_{apo}^{folding\text{-}ZFP} = \Delta G^{ML\text{-}obs\text{-}\mathbf{ZFP}} - \Delta G^{ML\text{-}obs\text{-}\mathbf{GGG}} \quad (4)$$

We,[13] and others,[14,15] have used this method to deduce the free energy cost of protein folding for natual zinc fingers. A typical zinc finger possesses a cost of protein folding between 0 and +4.2 kcal/mol that is minimal compared to the −18 kcal/mol contribution of Zn(II) binding. The critical assumption in this analysis, i.e., that the cost of protein folding in the **GGG** series of peptides is 0 kcal/mol, has been called into question by Sénèque and Latour[22] who have reported substantially tighter Zn(II) binding constants for Berg's CP1[23−25] series of designed zinc fingers. Furthermore, they observe slow equilibration kinetics for the removal of Zn(II) from CP1 peptides containing His ligands suggesting a kinetic barrier to metal-ion removal. Their data suggest that it costs up to +5.8 kcal/mol to fold the **GGG** peptide which in turn raises the cost of protein folding in zinc fingers to +6 to +11 kcal/mol.

In order to validate the key assumption of our analysis, we chose to determine the formation constant of a natural Zn(II) protein that is folded in both the *apo*- and *holo*-forms, has a canonical zinc finger coordination motif, and shows facile metal-ion exchange kinetics. The 59 amino acid zinc binding domain of human transcription factor IIB (ZBD-TFIIB) satisfied these criteria and was chosen for study.[26] Figure 1 shows the NMR solution structures of Zn(II)-ZBD-TFIIB and *apo*-ZBD-TFIIB which possess the same zinc ribbon fold[17] with the Cys_3His_1 ligand set displayed in a rubredoxin-knuckle. The

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backbone structures of *apo*- and *holo*-ZBD-TFIIB are essentially identical. However, the *β*-turn residues, Arg^{28}–Gly^{30}, are disordered in the *apo*-state structure. Since the structures *apo*- and *holo*-ZBD-TFIIB are virtually equivalent, metal-ion binding does not drive protein folding, and $\Delta G^{ML\text{-}obs}$ is equivalent to ΔG^{ML} because of the lack of a metal-induced protein folding as shown in Scheme 1. Herein, the inherent pH-independent formation constant for Zn(II)-ZBD-TFIIB is determined using equilibrium thermodynamic measurements over the pH range 4.5–9.0 followed by fluorescence spectroscopy and isothermal titration calorimetry. The Zn(II)-ZBD-TFIIB data are compared to natural zinc finger proteins to reveal the cost of protein folding in the latter.

Isothermal Titration Fluorimetry. The 355 nm fluorescence emission maximum, λ_{max}^{em}, of *apo* and *holo* ZBD-TFIIB is indicative of a solvent exposed tryptophan and does not shift wavelength with Zn(II) incorporation, consistent with its position near the flexible C-terminus and the lack of hydrophobic burial upon Zn(II) coordination. Zn(II) binding results in an enhancement of the fluorescence emission of the native tryptophan; there is a ~10% increase in fluorescence emission intensity upon metal incorporation. This is presumably due to the loss of quenching from the cysteine thiols upon Zn(II) binding as thiolates.[27] The change in fluorescence is smaller than observed in the **GGG** peptide series, and in the C-terminal zinc finger of the human Wilms Tumor Suppressor protein, **WT1–4**, studied in this laboratory,[14] due to the greater distance between the quenching thiols and the tryptophan residue.

Fluorescence spectroscopy was employed to characterize the pH dependence of the thermodynamics of Zn(II) binding to ZBD-TFIIB over the pH range 4.5–9.0 because Zn(II) binding is coupled to proton release. Figure 2 shows the direct titration

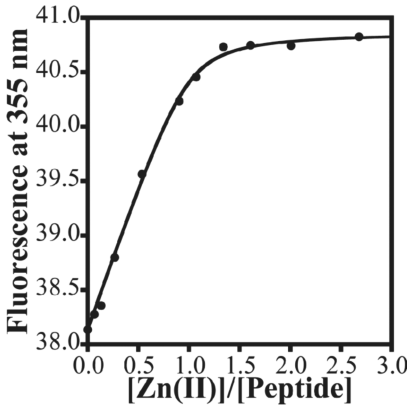

Figure 2. Fluorescence determination of the Zn(II)-ZBD-TFIIB conditional dissociation constant by direct titration of $Zn(II)Cl_2$ in unbuffered aqueous solution at pH 7.0 into 10 *μ*M ZBD-TFIIB buffered at pH 5.5 (20 mM MES, 100 mM KCl). The increase in emission intensity at 355 nm observed upon Zn(II) binding is fit in the inset to a Zn(II)-ZBD-TFIIB conditional dissociation constant, K_d, value of 1.0 *μ*M at pH 5.5.

of Zn(II) into *apo*-ZBD-TFIIB at pH 5.5 (20 mM MES, 100 KCl) followed by fluorescence under strictly anaerobic conditions. Kinetic measurements indicated equilibrium is reached in <3 min, and the samples were allowed to equilibrate for at least 10 min. The increase in fluorescence emission at 355 nm is adequately fit to a 1:1 metal/protein equilibrium binding model with a K_d value of 1.0 *μ*M (*μ*M) at pH 5.5. This value is identical within error to the value determined by isothermal titration calorimetry (*vide infra*). Furthermore, this value is less than 1 kcal/mol tighter, and within the experimental error, of the 5.0 *μ*M value measured for Zn(II)-**GGG**-Cys_3His_1 at pH 5.5.[13]

Binding constant determinations under more basic conditions required the use of the competitors EDTA and HEDTA.[9] Figure 3 shows a representative equilibrium

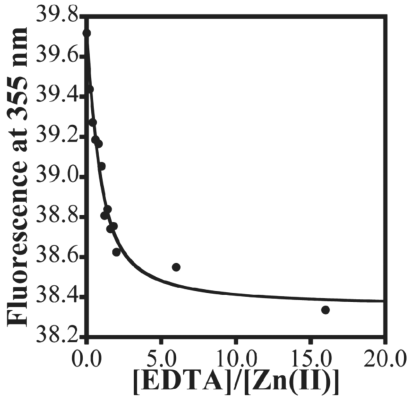

Figure 3. Fluorescence determination of the Zn(II)-ZDB-TFIIB conditional dissociation constant at pH 9.0 (20 mM TRIS, 100 mM KCl) by competition titration with EDTA. An aqueous solution containing 20 *μ*M ZBD-TFIIB and 10 *μ*M Zn(II) was titrated with 0–16 equiv of EDTA buffered at pH 9.0. Under these conditions, a fit to the plot of fluorescence at 355 nm vs equivalents of EDTA added to peptide gives a competition constant value of 2.0 between Zn(II)-ZBD-TFIIB and EDTA. Since the K_d of Zn(II)-EDTA at pH 9.0 is 600 aM, the resulting Zn(II)-ZBD-TFIIB dissociation constant at pH 9.0 is 300 aM.

competition titration between EDTA and Zn(II)-ZBD-TFIIB at pH 9.0 (20 mM TRIS, 100 KCl). Kinetic measurements indicated equilibrium is established in <5 min, and the samples were allowed to equilibrate for at least 15 min. The resulting competition isotherm shows a loss in fluorescence emission intensity due to transfer of the Zn(II) from Zn(II)-ZDB-TFIIB to EDTA. The data are fit to an equilibrium competition expression that indicates a K_{comp} value of 2.0 and a Zn(II)-ZDB-TFIIB K_d value of 300 attomolar (aM) at pH 9.0. This value is less than 0.5 kcal/mol tighter, and within the experimental error, of the 700 aM K_d value measured for Zn(II)-**GGG**-Cys_3His_1 at pH 9.0. Indeed, all the a Zn(II)-ZDB-TFIIB K_d values measured are within 1 kcal/mol of the reported values for Zn(II)-**GGG**-Cys_3His_1 at the same pH value.[13] This indicates not only a similar overall formation constant, but also a similar pH dependence, in other words, identical intrinsic and effective Cys and His pK_a values.

Isothermal Titration Calorimetry. The individual entropic and enthalpic contributions for Zn(II)-ZBD-TFIIB formation were determined using ITC. The ITC was studied at three pH values (5.5, 7.0, and 8.0) since Zn(II) binding is pH dependent. At each pH value, the observed enthalpy (ΔH_{obs}) was measured in three buffers to determine the number of protons released and corrected to yield the reaction enthalpy (ΔH_{obs}). Figure 4 shows the direct titration of Zn(II) into 25 *μ*M *apo*-ZBD-TFIIB at pH 5.5 (20 mM MES, 100 KCl) followed by isothermal titration calorimetry under strictly anaerobic conditions. These data indicate a K_d value of 0.5 *μ*M at pH 5.5, a value identical within error to that measured using

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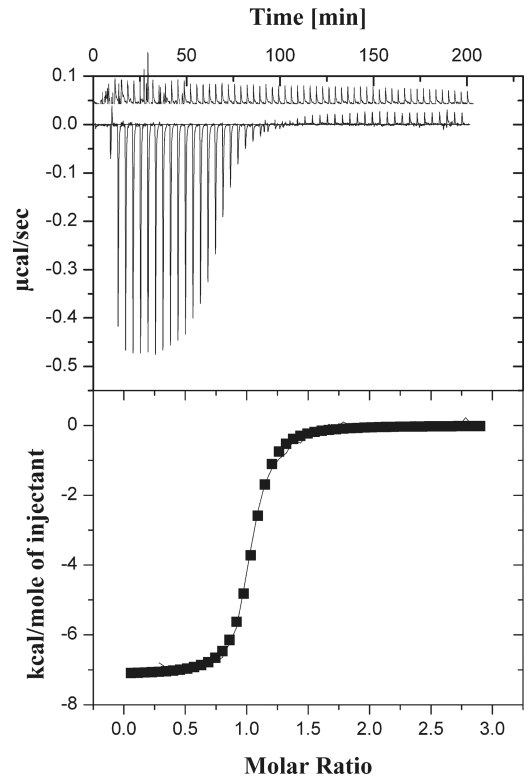

Figure 4. Thermogram and equilibrium binding isotherm of $ZnCl_2$ titrated into ZBD-TFIIB at pH 5.5 (20 mM MES, 100 KCl). A 1:1 binding model fit to the isotherm indicates a K_d value of 0.5 μM at pH 5.5, a value identical to that obtained by fluorimetric titration.

fluorescence spectroscopy (*vide supra*). In addition, ITC showed that 2.0 protons were released upon Zn(II), consistent with the results from Zn(II)-**GGG**-Cys_3His_1 at pH 5.5.[13] ITC measurements conducted at pH 7.0 and 8.0, combined with the K_d values determined by fluorescence, demonstrate the modulation of the Zn(II) affinity by solution pH, Figure 5. Table 1 shows that Zn(II) binding to ZBD-TFIIB is entropically driven at pH 5.5 and 7.0, while at pH 8.0 Zn(II) binding is both entropically and enthalpically favorable.

Equilibrium Binding Model. The minimal complete equilibrium binding model established for **GGG**-Cys_3His_1, presented in Scheme 2, was used to determine the formation constant for Zn(II)-ZBD-TFIIB due to the similarity in the pH dependence of their measured conditional dissociation constants and the numbers of protons released in the ITC measurements. The model incorporates five *apo*-state species, generically LH_4 to L^{4-}, due to the stepwise deprotonation of the His and Cys residues with pK_a values close to the amino acid solution values. In the *holo*-state, the model has a cooperative three-proton cysteine deprotonation event, pK_{a2-4}^{eff}, which results in only three *holo*-state species, i.e., MLH_4^{2+}, MLH_3^{+}, and ML^{2-} where MLH_4^{2+} dissociates into M(II) and LH_4 under the conditions of our experiment. The pH dependence of the formation constant is given by eq 1.

DISCUSSION

A suite of coordination chemistry equilibrium measurements have been used to determine the formation constant for the natural Cys_3His_1 zinc binding domain of human transcription factor IIB. The data, fit to a minimal complete equilibrium model for metal−peptide binding, demonstrate the metal-ion

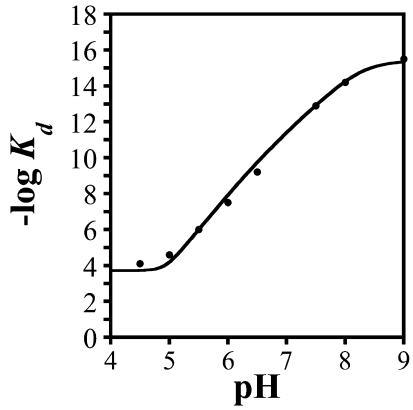

Figure 5. pH dependence of the conditional dissociation constant of Zn(II) complexation by ZBD-TFIIB, shown as a plot of the logarithm of the association constant vs solution pH. The equilibrium binding model employed to fit the data yields a pH-independent formation constant, K_f^{ML}, value of 1.5×10^{15} M^{-1}, or a limiting dissociation constant of 700 attomolar, which corresponds to a reaction free energy of −21.1 kcal mol^{-1}.

Table 1. Thermodynamics of Zn(II)-ZBD-TFIIB at 298 K

	pH 5.5	pH 7.0	pH 8.0
K_d	0.5 μM	1.5 pM	3.5 fM
ΔG_{rxn} (kcal/mol)	−8.6	−16.1	−19.7
ΔH_{rxn} (kcal/mol)	+0.6	+0.2	−4.5
$-T\Delta S_{rxn}$ (kcal/mol)	−9.2	−16.3	−15.2
H^+ released	2.0	3.2	2.3

affinity and solution speciation of Zn(II)-ZBD-TFIIB along with a formation constant value of 1.5×10^{15} M^{-1}, and a limiting K_d value of 700 aM. Since ZBD-TFIIB retains the same overall three stranded β-sheet structure in both the *apo*- and *holo*-states,[17] the observed free energy of metal-ion binding, $\Delta G^{ML\text{-}Obs}$, is equivalent to the intrinsic metal binding free energy, ΔG^{ML}. The data demonstrate that Zn(II) binding to a Cys_3His_1 coordination sphere without significant protein folding effects is favorable by −21.1 kcal/mol and modulated by solution pH.

In terms of formation constants, the 1.5×10^{15} M^{-1} K_f^{ML} value of Zn(II)-ZBD-TFIIB (limiting K_d value of 700 aM), which is well-folded and isostructural in both *apo*- and *holo*-states, reveals, for the first time, the inherent free energy of Zn(II)-Cys_3His_1 interactions independent of metal-induced protein folding effects. The K_f^{ML} values of Zn(II)-ZBD-TFIIB and Zn(II)-**GGG**-Cys_3His_1, our simple, unstructured model peptide,[13] are identical at 1.5×10^{15} M^{-1}. These values are also identical within error to the 2×10^{15} M^{-1} value measured for amino acids 34−51 of HIV-1 nucleocapsid protein, (34−51)NCp7,[26] a CCHC zinc finger peptide. Since the free energy contribution of Zn(II) binding to the Cys_3His_1 sites in ZBD-TFIIB, **GGG**-Cys_3His_1, and (34−51)NCp7[26] are identical, the free energy cost of protein folding is the same in each, i.e., zero kcal/mol.

These three K_f^{ML} values are all ∼5000-fold weaker than the 7.9×10^{18} M^{-1} value for Zn(II)-CP1-(CCHC) reported by Sénèque and Latour[22] during a re-evaluation of Zn(II)-CP1 binding interactions. Since ZBD-TFIIB is prefolded prior to Zn(II) binding, and CP1-(CCHC) possesses a metal-induced protein folding event ($\Delta G_{apo}^{folding} \geq 0.0$ kcal/mol), the binding of Zn(II) to CP1-(CCHC) is not expected to be tighter than

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Table 2. Cost of Protein Folding in Natural Zinc Fingers

		Zn(II)-protein		Zn(II)-ZDB-TFIIB			
protein (AA)	pH	K_d	$\Delta G^{ML\text{-}obs}$	K_d	$\Delta G^{ML\text{-}obs}$	$\Delta G_{apo}^{folding}$	protein fold
L36[28]	6.0	17 nM	−10.6[a]	18 nM	−10.6	0.0	3 stranded β-sheet
NZF-1[29]	6.9	125 pM	−13.5[a]	9 pM	−15.0	+1.5	unknown
$RMLV_{protein}$[30]	7.0	1 pM	−16.4[a]	4pM	−15.5	−0.9	random coil
$RMLV_{peptide}$[31]	7.0	690 pM	−12.5[a]	4pM	−15.5	+3.0	random coil
MyT1−2[15]	7.4	90 nM	−9.4[a]	213 fM	−17.3	+7.9	unstructured
(35−50)NCp7[27]	7.5	312fM	−17.0[a]	125 fM	−17.6	+0.6	β-turns
$MoMuLV_{protein}$[32]	7.9	6.0 fM	−19.4[a]	8.5 fM	−19.2	−0.2	random coil
Fw[33]	8.0	1.9 pM	−16.0[a]	6.3 fM	−19.4	+3.4	unknown
(34−51)NCp7[27]	9.0	500 aM	−20.9[a]	300 aM	−21.2	+0.3	random coil

[a]In kcal/mol.

that of ZBD-TFIIB. While Berg and co-workers[23] did not measure the K_f^{ML} value of Zn(II)-CP1-(CCHC), their reported K_d values are comparable to the K_d values reported here for Zn(II)-ZBD-TFIIB at the same pH values, and those previously reported for Zn(II)-**GGG**-Cys_3His_1 and (34−51)NCp7. Thus, the Zn(II)-ZBD-TFIIB data support the thermodynamic findings of Berg over those of Sénèque and Latour for Zn(II)-CP1-(CCHC). In terms of kinetics, we note that the kinetics of metal-ion removal from Zn(II)-ZBD-TFIIB and Zn(II)-**GGG**-Cys_3His_1 by EDTA are complete within 10 min. These kinetics are similar to those of Co(II)/Zn(II) metal-ion exchange reported by Berg for a CP1-(CCHH),[25] and distinct from the reported >16 h equilibration times reported by Sénèque and Latour for EDTA removal of Zn(II) from Zn(II)-CP1-(CCHC).[22] Thus, our data support the thermodynamic and kinetic findings of Berg but do not address the source of the discrepancy in the Zn(II)-CP1-(CCHC) data.

In terms of conditional binding constants, a comparison of the Zn(II)-ZBD-TFIIB data to those of the Zn(II)-**GGG**-Cys_3His_1 model peptide validate our original assumption that the cost of protein folding in **GGG** was close to 0 kcal/mol. The conditional K_d values of Zn(II)-ZBD-TFIIB and Zn(II)-**GGG**-Cys_3His_1 are equivalent, within the 1 kcal/mol error of the measurements, at all pH values studied, as are the overall formation constant values of 1.5×10^{15} M^{-1}. Furthermore, the numbers of protons released at pH 5.5, 7.0, and 8.0 determined by ITC measurements in different buffers are equivalent between the two proteins indicating equivalent intrinsic and effective pK_a values of the Cys and His ligands. Since Zn(II)-ZBD-TFIIB is virtually isostructural in the *apo*- and *holo*-states, its cost of protein folding is zero kcal/mol, and therefore, the cost of protein folding in Zn(II)-**GGG**-Cys_3His_1 must also be zero kcal/mol. A comparison of the K_d values of Zn(II)-ZBD-TFIIB with those from natural Cys_3His_1 zinc finger peptides reveals the cost of protein folding in the latter. As shown in Table 2, $\Delta G_{apo}^{folding}$ values are <+3.5 kcal/mol in all but one case and much less than the free energy derived from Zn(II)-Cys_3His_1 interactions for proteins ranging in size from 16 to 56 amino acids and with various global folds.[28−33] The outlier with a +7.9 kcal/mol cost of protein folding, human myelin transcription factor 1 (MyT1-2), does not possess any secondary structure in the folded state.[15] Since any favorable protein−protein interactions (hydrogen bonds, hydrophobic interactions, etc.) stabilize the folded states (both *apo* and *holo*), the lack of these favorable protein−protein interactions from negligible secondary structure formation in MyT1-2 results in a higher value of $\Delta G_{apo}^{folding}$ than that observed in the other zinc proteins. The minimal $\Delta G_{apo}^{folding}$ values observed for most zinc finger proteins suggest that the role of Zn(II) is not to thermodynamically drive protein folding, but perhaps rather to kinetically template protein folding and/or modulate protein dynamics.

The conclusion that the cost of protein folding in zinc fingers is minimal is suggested by literature precedence. Most recently, Pavone and co-workers have discovered MI3 and MI4, natural homologues of the prokaryotic $Cys_2His_2/\beta\beta\beta\alpha\alpha$ zinc finger protein, Ros87, which lack Zn(II) yet achieve the same functional fold.[34,35] This suggests a minimal cost of protein folding in the same way that Mayo's demonstration that hydrophobic interactions can be used to compensate for the removal of the Zn(II) ligands and provide a stably folded zinc finger $\beta\beta\alpha$ structure does.[36] Imperiali and co-workers also showed that the redesign of a $\beta\beta\alpha$ zinc finger into a folded *apo*-state did not have the dramatic impact on the Zn(II) affinity expected on the basis of the assumption of a large unfavorable cost of protein folding.[37,38] Lastly, Pande and co-workers have put forth the mean structure hypothesis from molecular dynamics simulations where they observe that the average *apo*-unfolded structure corresponds to the native folded state in an average sense.[39] All of these studies support our conclusion that the cost of protein folding in natural zinc fingers is minimal compared to the free energy contribution of Zn(II) binding.

Zn(II) binding to zinc finger proteins is an entropically favored process in many, but not all, cases.[13,15,40] Table 1 shows that Zn(II) binding to *apo*-ZBD-TFIIB is entropically driven at the two pH values studied (5.5, 7.0) and entropically and enthalpically favored at pH 8.0. The observed favorable reaction entropy values are due to dissociation of protons from the Cys/His ligands and the dissociation of water from both the $Zn(II)(H_2O)_6^{2+}$ and the *apo*-protein reactants. The reaction is enthalpically disfavored at pH 5.5 and 7.0, but enthalpically favored at pH 8.0. The observed trend in ΔH values as a function of pH tracks with protons released from the amino acid ligands, and includes contributions from the release of water molecules bound to $Zn(II)(H_2O)_6^{2+}$ and associated with the protein scaffold. Interestingly, the data for Zn(II)-ZBD-TFIIB are similar to our previously reported data for Zn(II)-**GGG**-Cys_3His_1 (at pH values of 5.5, 7.0, and 8.0), with the Zn(II)-ZBD-TFIIB reaction being slightly more unfavorable enthalpically and more favorable entropically. Since the exact magnitudes of ΔH and $-T\Delta S$ contain contributions from protein−protein interactions, literature values vary considerably by zinc protein scaffold. For example, the ΔH and $-T\Delta S$ values Zn(II) binding to the Cys_3His_1 site in HIV-1 nucleocapsid protein (+6.4 and −21.7 kcal/mol, respectively, at pH 7.0)[40] and in the second zinc finger of myelin transcription factor (−2

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and −7.5 kcal/mol at pH 7.4)[15] are modulated by their respective protein folding events, but $-T\Delta S$ remains the largest contributor to their reaction free energies.

CONCLUSION

In toto, we have determined the formation constant for Zn(II)-ZBD-TFIIB to determine the intrinsic free energy contribution of Zn(II) binding a Cys_3His_1 coordination sphere to assess the free energy cost of protein folding in zinc finger proteins with metal-induced protein folding events. Since it is essentially isostructural in the *apo*- and *holo*-states, a comparison of the zinc conditional dissociation constants of Zn(II)-ZBD-TFIIB to Cys_3His_1 zinc fingers reveals that the cost of protein folding in the latter is minimal relative to the favorable free energy of Zn(II) binding. The data are fully consistent with Berg's thermodynamic and kinetic data on CP-1(CCHC) as well as our own data on **GGG**-Cys_3His_1. Our efforts are now focused on evaluating the role of zinc in kinetically templating protein folding and modulating structural dynamics in zinc finger proteins.

ASSOCIATED CONTENT

Supporting Information

HPLC chromatograms of crude and pure ZBD-TFIIB and mass spectrometric analysis. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.inorgchem.5b00718.

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Notes

The authors declare no competing financial interest.

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