Ligand Variation and Metal Ion Binding Specificity in Zinc Finger Peptides†

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Three metal binding peptides with coordination sites Cys_2His_2, Cys_3His, and Cys_4 have been prepared and their metal binding properties characterized. The peptides are based on a zinc finger consensus sequence and have the sequences ProTyrLysCys$_4$ProGluCys$_7$GlyLysSerPheSerGlnLysSerAspLeuValLysXaa$_{20}$GlnArgThrYaa$_{24}$ThrGly (**Xaa = Yaa = His**; **Xaa = His, Yaa = Cys**; **Xaa = Yaa = Cys**). The dissociation constants for the peptide complexes with Co^{2+}, Zn^{2+}, and Cd^{2+} have been determined via a series of direct and competitive metal ion titrations. The trend in relative affinities of the peptides for Co^{2+} over Zn^{2+} can be semiquantitatively accounted for by the decrease in ligand field stabilization energy as imidazole ligands are replaced by thiolates. The affinity for Cd^{2+} increases by over two orders of magnitude for each thiolate for imidazole substitution, in keeping with hard–soft acid–base effects. Furthermore, the results reveal that the N_2S_2 coordination site is unique among the sites studied in allowing significant preferential binding of Zn^{2+} over both first row transition metals and second row elements such as Cd^{2+}.

Introduction

The ability of certain molecules to bind specifically one of a closely related set of species is fundamental to biology and to the developing area of molecular recognition chemistry. One particular case involves metal ion recognition. Metal-requiring proteins must bind their cognate metal ions with appropriate affinity for correct biosynthesis or response to changes in free metal ion concentration. The zinc finger proteins,[1-5] typified by *Xenopus* transcription factor IIIA, are specifically activated by zinc preferentially over various divalent first row transition metal ions,[1,6-8] and this preference is reflected in the ability of single zinc finger peptides to bind these ions.[9,10] A major determinant of the metal ion specificity is expected to be the nature of the metal binding residues. We now report studies of zinc finger peptides which have coordination sites of the types 2-Cys, 2-His; 3-Cys, 1-His; and 4-Cys. Metal binding studies using Co^{2+} and Cd^{2+}, as well as Zn^{2+}, reveal trends that show the importance of ligand field stabilization energy and hard-soft acid-base effects in determining metal ion specificity. These quantitative results should be useful in understanding the specificity of other metal-binding proteins and in designing novel metal-binding peptides with desired properties.

We have studied sequence variants of CP-1, a 26 amino acid peptide based on the consensus sequence of 131 zinc finger domains.[10] This peptide has the sequence ProTyrLysCys$_4$-ProGluCys$_7$GlyLysSerPheSerGlnLysSerAspLeuValLysHis$_{20}$-GlnArgThrHis$_{24}$ThrGly. The variants studied were CP-1(His_{24} to Cys) and CP-1(His_{20} to Cys, His_{24} to Cys), hereafter termed CP-1(CCHC) and CP-1(CCCC). Previous studies of CP-1 revealed that this peptide binds Co^{2+} and Zn^{2+} with dissociation constants of approximately 50 nM and 2 pM, respectively. These results demonstrated the large discrimination that these peptides can show between different metal ions. Furthermore, initial studies of CP-1(CCHC) revealed that ligand variation was possible by simple modification of the amino acid sequence without large loss in stability.

Experimental Section

Peptides were synthesized and purified as described previously.[10] All manipulations of the peptides were performed under an atmosphere of 95% nitrogen-5% hydrogen to avoid peptide oxidation. Spectroscopic studies were performed on Hewlett-Packard 8451 or Perkin-Elmer Lambda 9 spectrophotometers. Metal binding studies were performed in 100 mM HEPES, 50 mM NaCl, pH 7.0 buffer. Near infrared spectra were recorded in 20 mM perdeuterated Tris-DCl buffer at pH 7.0. The dissociation constant for the Cd^{2+} complex of CP-1 was determined by titrating a solution of Co^{2+} complex of CP-1 with Cd^{2+}. The bleaching of the absorption spectrum of the Co^{2+} complex was monitored and used to fit the dissociation constant as described previously.[9]

The relative affinities of pairs of peptides for Co^{2+} were determined by titrating an approximately equimolar mixture of the two peptides with Co^{2+} and monitoring the absorption spectrum. The data from 500 to 800 nm were deconvoluted using the previously measured spectra of the two peptide-Co^{2+} complexes to determine the concentrations of the two complexes. The relative affinities of pairs of peptides for Zn^{2+} and Cd^{2+} were determined by titrating the Co^{2+} complexes of the two peptides with either Zn^{2+} or Cd^{2+}. The decrease in absorbance of the Co^{2+} complex was monitored and used to calculate the relative affinities.

The deconvolution was performed by representing each spectrum as a vector $S = (A_{500}, A_{501}, ..., A_{800})$ where A_i is the absorbance at wavelength i. The spectra of the two pure components were represented similarly as vectors $C_{1,2}$. These were converted to an orthogonal basis set $B_{1,2}$ where $B_1 = C_1$ and $B_2 = C_2 - rC_1$ where $r = (C_1 \cdot C_2)/(C_1 \cdot C_1)$. This basis set was used to determine the deconvolution of S in terms of C_1 and C_2, i.e. $S = k_1C_1 + k_2C_2$ where $k_1 = z_1 - rz_2$ and $k_2 = z_2$ with $z_1 = (S \cdot B_1)/(B_1 \cdot B_1)$ and $z_2 = (S \cdot B_2)/(B_2 \cdot B_2)$. The values of k_1 and k_2 were converted to fractional saturation values Y_1 and Y_2 by dividing by the total concentration of the appropriate component.

These fractional saturation data were used to determine the ratio of dissociation constants for the two peptides for a given metal. The ratio of dissociation constants K_{d1}/K_{d2} can be expressed in terms of the fraction saturation values as $c = K_{d1}/K_{d2} = (Y_2(1 - Y_1))/(Y_1(1 - Y_2))$. This can be solved for the difference in fractional saturation $Y_1 - Y_2 = ((1 + c)$

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† Dedicated to Professor Richard H. Holm on the occasion of his 60th birthday.

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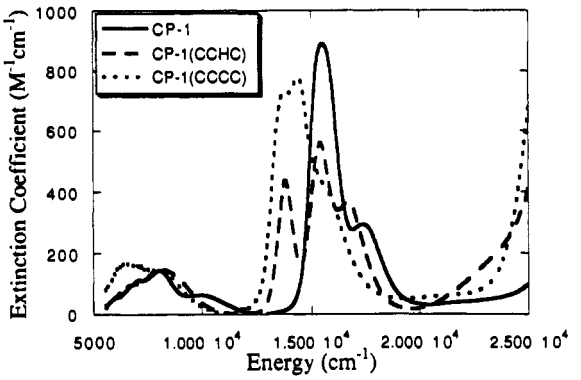

Figure 1. Absorption spectra of the Co^{2+} complexes of CP-1, CP-1(CCHC), and CP-1(CCCC) in the visible and near-infrared regions. The less intense bands between 5000 and 10000 cm^{-1} correspond to $^4A_2$ to $^4T_1(F)$ transitions whereas the more intense bands between 12000 and 18000 cm^{-1} correspond to $^4A_2$ to $^4T_1(P)$ transitions.[13] Note that both sets of bands shift to lower energies as the number of thiolate ligands increases.

Table I. Spectroscopic Parameters for the Co^{2+} Complexes of CP-1, CP-1(CCHC), and CP-1(CCCC)[a]

peptide	ν_2 (cm^{-1})	ν_3 (cm^{-1})	Δt (cm^{-1})	B (cm^{-1})
CP-1	8300	15900	4930	627
CP-1(CCHC)	7900	15400	4675	618
CP-1(CCCC)	7400	14600	4370	592

[a] The values of ν_2 and ν_3 correspond to the energies of the $^4A_2$ to $^4T_1(F)$ and $^4A_2$ to $^4T_1(P)$ transitions, respectively. These energies were determined from the centers of mass of the absorption peaks shown in Figure 1. The values of Δt and B were calculated via the equations[12] $(\Delta t)^2 - 0.529(\nu_2 + \nu_3)\Delta t + 0.294\nu_2\nu_3 = 0$ and $B = (1/15)(\nu_2 + \nu_3 - 3(\Delta t)$. The estimated uncertainties for the Δt values are ±100 cm^{-1}.

$- ((x-1)^2(1-c)^2 + 4c))^{1/2}/(1-c)$ where $x = Y_1 + Y_2$. The data in the form of $Y_1 - Y_2$ versus $Y_1 + Y_2$ were fit using this expression to yield c with use of the program Kaleidagraph (Abelbeck Software). The same analysis applies to the experiments with two metal ions except that c is replaced by the ratio of the ratio of dissociation constants $(K_d^{M_1}/K_d^{M_2})_1/(K_d^{M_1}/K_d^{M_2})_2$. This was used for experiments in which the peptides were first saturated with Co^{2+} and then back-titrated with Zn^{2+} or Cd^{2+}.

Results and Discussion

The absorption spectra of the Co^{2+} complexes of the three peptides in the visible and near-infrared regions are shown in Figure 1. The d–d transitions shift to lower energies as the number of cysteinate ligands increases from two to three to four. The energies of these transitions and the corresponding values of Δt are given in Table I. As expected from the positions of imidazole and thiolate in the spectrochemical series,[11,12] Δt decreases approximately linearly as imidazoles are replaced by thiolates. Direct titration of these peptides with Co^{2+} yielded data that could be fit with dissociation constants of $< 10^{-7}$, $< 10^{-7}$, and $3.5 (\pm 1.0) \times 10^{-7}$ for CP-1, CP-1(CCHC), and CP-1(CCCC), respectively. Only upper bounds could be determined for the first two dissociation constants due to the tight binding of Co^{2+} by these peptides.

The differences in the absorption spectra allow titrations to be performed in which a mixture of peptides is titrated with Co^{2+} and the resulting spectra are deconvoluted to yield the fractional saturations of the two peptide sites. Such competition experiments allow much more precise determination of relative metal ion affinities than could be determined by separate titration experiments. The spectra for a titration of a mixture of CP-1 and CP-1(CCHC) are shown in Figure 2 and a representative deconvolution is shown in Figure 3. The data from such experiments can be fit to determine the ratio of the metal-peptide complex dissociation constants as described in the Experimental Section. Furthermore, similar methods can be used to study the relative specificities of two peptides for different metal ions. In this case, the peptides are saturated with one metal, Co^{2+}, and the mixture is back-titrated with another metal ion such as Zn^{2+} or Cd^{2+}. The results of these experiments for CP-1 and CP-1(CCCC) are displayed in Figure 4. The relative dissociation

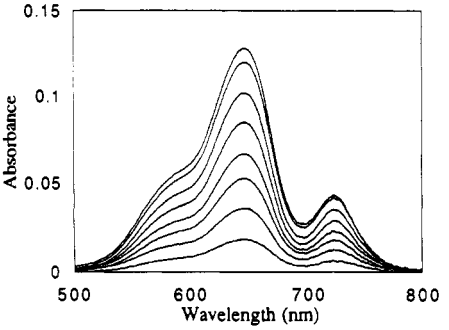

Figure 2. Absorption spectra of a Co^{2+} titration containing approximately equimolar amounts of CP-1 and CP-1(CCHC).

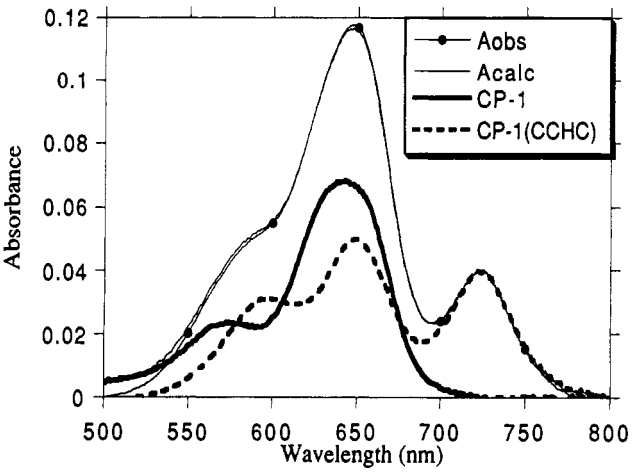

Figure 3. Deconvolution of one of the Co^{2+} spectra from the CP-1 and CP-1(CCHC) combined titration shown in Figure 2. The experimental spectra, shown with a thin line and filled circles, was fit using the deconvolution program described in the Experimental Section. The calculated spectra due to the CP-1 and CP-1(CCHC) complex components are shown in bold lines.

Table II. Relative Dissociation Constants[a]

peptide	$(K_d^{Co})_{rel}$	$(K_d^{Co}/K_d^{Zn})_{rel}$	$(K_d^{Zn})_{rel}$	$(K_d^{Cd}/K_d^{Zn})_{rel}$	$(K_d^{Cd})_{rel}$
CP-1	1	1	1	1	1
CP-1(CCHC)	1.0	1.8	0.56	3.2×10^2	3.2×10^{-3}
CP-1(CCCC)	5.6	30	0.19	2.8×10^5	2.0×10^{-5}

[a] The relative dissociation constants were determined as described in the Experimental Section. The estimated uncertainties for the relative dissociation constants are ±20%.

Table III. Absolute Dissociation Constants (M)[a]

peptide	K_d^{Co}	K_d^{Zn}	K_d^{Cd}
CP-1	$6.3 (\pm 2.2) \times 10^{-8}$	$5.7 (\pm 1.3) \times 10^{-12}$	$2.0 (\pm 1.5) \times 10^{-9}$
CP-1(CCHC)	$6.3 (\pm 2.2) \times 10^{-8}$	$3.2 (\pm 1.0) \times 10^{-12}$	$6.4 (\pm 5.0) \times 10^{-12}$
CP-1(CCCC)	$3.5 (\pm 1.0) \times 10^{-7}$	$1.1 (\pm 0.3) \times 10^{-12}$	$4.0 (\pm 3.1) \times 10^{-14}$

[a] The absolute values of the Co^{2+} dissociation constants were determined using the ratios in Table II and the absolute value of the dissociation constant for CP-1(CCCC)-Co^{2+}. Because CP-1(CCCC) binds Co^{2+} somewhat more weakly than CP-1 and CP-1(CCHC), the titration data for CP-1(CCCC) could be reliably fit by nonlinear least-squares methods. The absolute values for the Zn^{2+} and Cd^{2+} dissociation constants were calculated using the ratios obtained earlier and the absolute values of K_d^{Zn} and K_d^{Cd} for CP-1 as determined by back-titrations of the CP-1 Co^{2+} complex. Note that these values differ slightly from those previously reported[10] because of improvements in data analysis methods.

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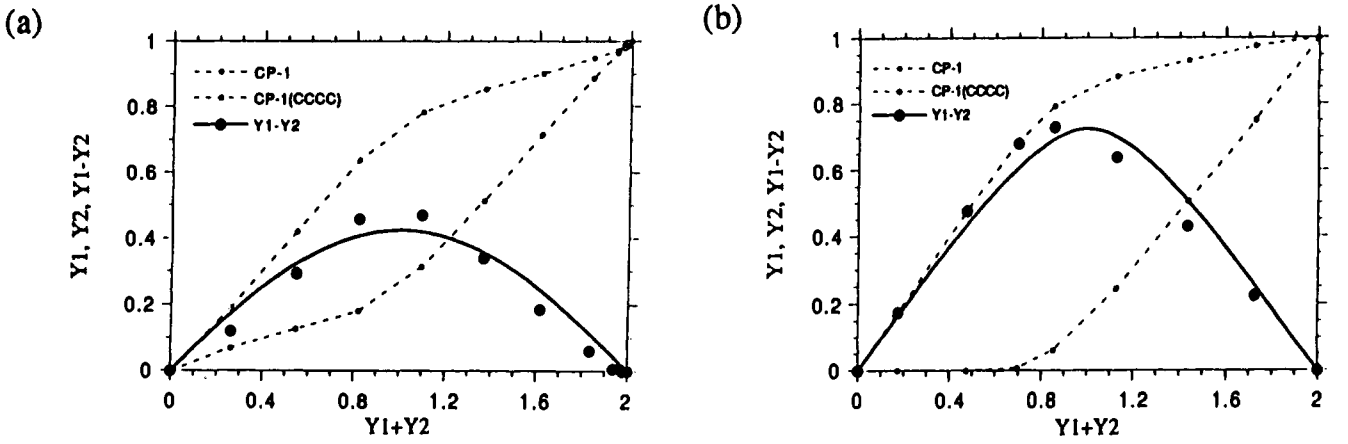

Figure 4. (a) Co^{2+} and (b) Co^{2+}/Zn^{2+} competition experiments between CP-1 and CP-1(CCCC). The difference in fractional saturation between the peptides ($Y_1 - Y_2$) was fit as described in the Experimental Section and used to calculate the relative affinities of the peptides for each metal. The fractional saturation of each peptide with metal is shown with dashed lines.

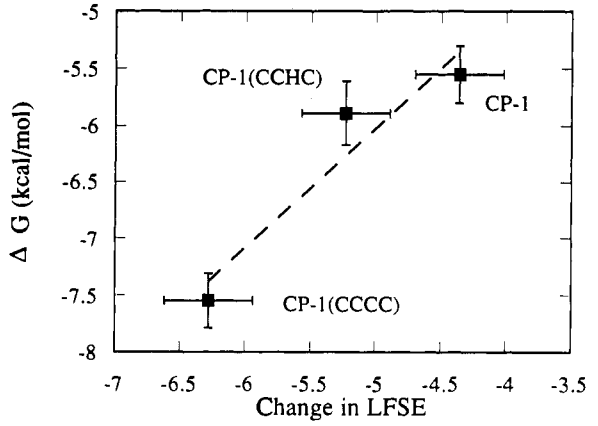

Figure 5. Comparison of ΔG and the ligand field stabilization energy (LFSE) for the Co^{2+}/Zn^{2+} exchange reaction (eq 1) for CP-1, CP-1(CCHC), and CP-1(CCCC).

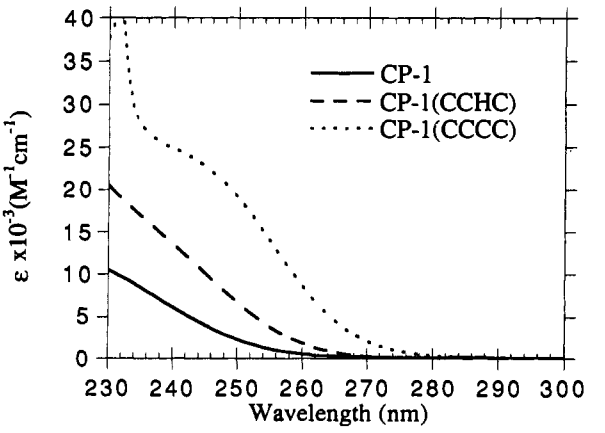

Figure 6. Absorption spectra of the Cd^{2+} complexes of CP-1, CP-1(CCHC), and CP-1(CCCC).

constants deduced from these experiments are summarized in Table II and the absolute dissociation constants are shown in Table III.

When a transition metal ion is bound in a site of nonspherical symmetry, the d orbitals split into sublevels, the lower of which are preferentially occupied. This leads to stabilization (by the ligand field stabilization energy, LFSE) over a hypothetical state in which the d orbitals have the same average energy but are uniformly occupied.[13] For a tetrahedral Co^{2+} complex, the LFSE is $-6/5\Delta t$ where Δt is the splitting between the e and t_2 sets of orbitals. We have previously noted that the difference between the LFSE for a Co^{2+} ion bound in a tetrahedral N_2S_2 site and that for Co^{2+} in an octahedral site in aqueous solution accounts for most of the free energy favoring Zn^{2+} binding to a zinc finger peptide. Thus, for the exchange reaction (1) a major contribution

$$\text{peptide–}Co^{2+} + Zn(OH_2)_6^{2+} \leftrightarrow \text{peptide–}Zn^{2+} + Co(OH_2)_6^{2+} \quad (1)$$

to the driving force is the LFSE change for Co^{2+}. Zn^{2+} is a d^{10} ion and has no LFSE in any site. This observation suggests that changes in the LFSE in the peptide site via changes in Δt should be reflected in the free energy of specificity for Zn^{2+} over Co^{2+}. As noted above, Δt can be changed by variation of the ligands bound to the metal. Replacement of a histidine residue by cysteine results in a decrease in Δt and, hence, in the LFSE for the peptide–Co^{2+} complex. This should increase the binding specificity of the peptide for Zn^{2+} over Co^{2+}. As shown in Table II, this is the case. A plot of ΔG for eq 1 versus the LFSE is shown in Figure 5. Fitting these data with linear relationship yields a slope of 1.06 and a correlation coefficient of 0.95. Constraining the line to pass through the origin increased the slope to 1.19 and reduced the correlation coefficient to 0.94. These observations indicate that LFSE changes can semi-quantitatively account for the increase in specificity for Zn^{2+} over Co^{2+} as the number of thiolates is increased. These results indicate that the specificity of a peptide with a tetrahedral metal binding site for Zn^{2+} over Co^{2+} can be tuned in a systematic way via changes in the ligating atoms. It should be noted that, in general, factors other than the changes in LFSE energy, such as ionic radius and polarizability, will affect metal ion binding specificity. For the particular case of Zn^{2+} versus Co^{2+}, the effective ionic radii are very similar (0.60 Å (Zn^{2+}), 0.58 Å (Co^{2+}))[14] and their polarizabilities are similar as judged by changes in formation constants of octahedral complexes as a function of ligand type.[15] Thus, the factors other than changes in LFSE will tend to be small for this case. Indeed, the high degree of similarity between Zn^{2+} and Co^{2+} make this a particular challenge for discrimination and our results reveal how LFSE effects can produce large and rationally adjustable determinants of metal ion binding specificity.

As an example of a case where LFSE can play no role, the relative affinities of the peptides for another d^{10} ion, Cd^{2+}, were determined. Each of the peptides was shown to bind Cd^{2+}. The spectra of the peptide-Cd^{2+} complexes are shown in Figure 6. The charge transfer band shifts towards the red and increases in

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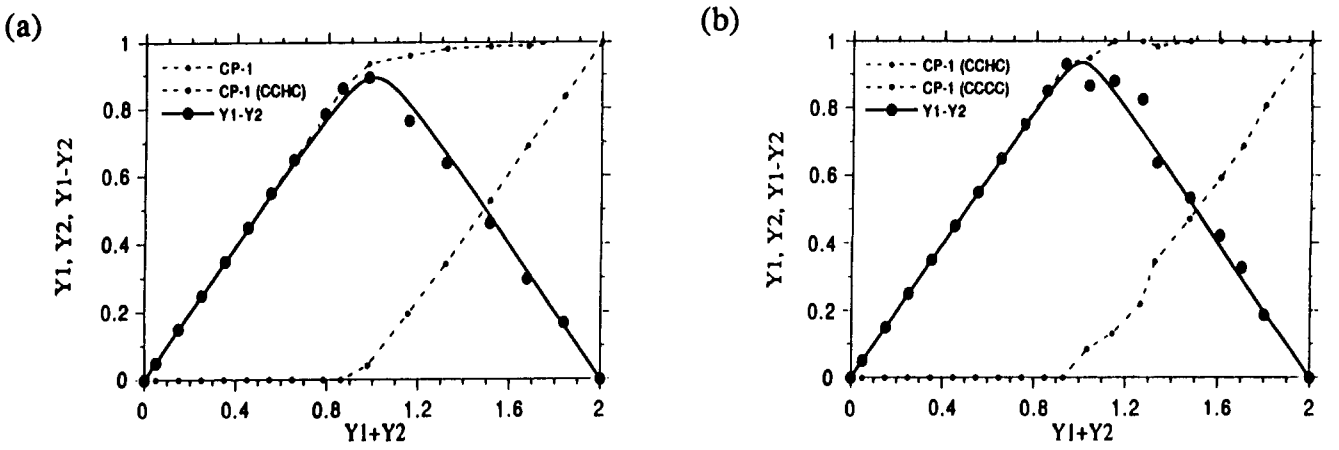

Figure 7. Cd^{2+} competition titrations between CP-1 and CP-1(CCHC) (a) and CP-1(CCHC) and CP-1(CCCC) (b). For both experiments the peptide with the higher number of cysteine ligands showed the greater affinity for Cd^{2+}, though there is a small amount of competitive binding by the other peptide. The difference in fractional saturation was fit as described earlier and used to calculate the relative affinities for Cd^{2+}.

apparent intensity as the number of thiolate ligands is increased. Experiments with CP-1 revealed that the spectrum changed shape in the course of the titration (data not shown). At low amounts of added Cd^{2+}, the spectrum resembled that seen for the complex of CP-1(CCCC) whereas it shifted to higher energies as the amount of Cd^{2+} was increased. These observations suggests that a 2:1 peptide Cd^{2+} complex forms at high peptide to metal ratios. Such behavior has been documented for variant zinc finger peptides.[16] In this case the driving force for 2:1 complex formation is the high affinity of Cd^{2+} for thiolate ligands. No changes in spectral shape were observed for the other peptides. Because of this complication and of the high affinities of these peptides for Cd^{2+}, affinities could not be determined by direct titration. Instead, competition methods were again used. Titration of a solution of CP-1 containing 2.5 equiv of Co^{2+} with Cd^{2+} resulted in bleaching of the visible spectrum of the Co^{2+} complex that could be fit with a dissociation constant of 2.0 ($\pm$1.5) $\times$ 10^{-9} M for the Cd^{2+} complex.

A dramatic increase in Cd^{2+} affinity was observed as the number of thiolate ligands was increased. Titrations were performed by adding Cd^{2+} to a mixture of peptides saturated with Co^{2+}. For CP-1 versus CP-(CCHC), Cd^{2+} showed a strong preference for CP-1(CCHC). This was also true for CP-1(CCHC) versus CP-1(CCCC) although the preference for the more thiolate-rich peptide was even stronger in this case. The data are shown in Figure 7 and the results summarized in Tables II and III. These observations are consistent with the hard–soft acid–base characteristics of these metal ions and peptides.[17] The soft metal Cd^{2+} binds the soft thiolate ligands preferentially over the imidazole ligands whereas the borderline metals Co^{2+} and Zn^{2+} show much less discrimination based on the hard–soft nature of the ligands. These observations are consistent with studies of metal binding proteins with all thiolate coordination sites which show stronger binding of Cd^{2+} than of Zn^{2+}.[18,19]

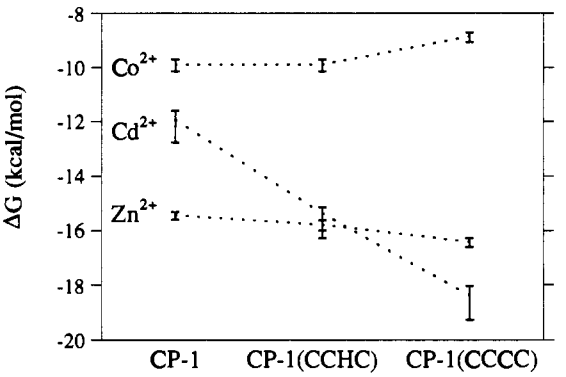

Figure 8. Summary of the free energies of Co^{2+}, Cd^{2+}, and Zn^{2+} binding for the three peptides CP-1, CP-1(CCHC), and CP-1(CCCC).

All of the binding data are summarized in Figure 8 in terms of free energies of binding versus ligand set. It is interesting to note that the natural ligand set allows preferential binding of Zn^{2+} over either Co^{2+} or Cd^{2+}. This discrimination also applies to Fe^{2+}, Ni^{2+}, and Mn^{2+}.[20] Given the central roles proteins of this class play in gene regulation during development and the possibility that changes in zinc levels are used to regulate protein activity, this may be a feature of considerable importance in the selection of the Cys_2His_2 ligand set. Studies of the effects of ligand variation on other properties such as the detailed three-dimensional structure and the ability of peptides to interact with nucleic acids are under investigation and will be reported in due course.

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