# Structure-Based Thermodynamic Analysis of a Coupled Metal Binding−Protein Folding Reaction Involving a Zinc Finger Peptide†

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ABSTRACT: The thermodynamics of metal binding by the prototypical $Cys_2His_2$ zinc finger peptide CP-1 has been examined through the use of isothermal titration calorimetry. In cholamine buffer at pH 7.0, the binding of zinc(II) to CP-1 shows an enthalpy change of $\Delta H°_{obs} = -33.7 \pm 0.8$ kcal/mol. Between one and two protons appear to be released accompanying the metal binding process. The heat of protonation of the cholamine buffer used is quite large (−11.5 kcal/mol), indicating that a portion of the observed metal binding enthalpy is due to buffer protonation. Structure-based thermodynamic analysis including the effect of water release from zinc(II) appears to account for the entropy associated with the coupled metal binding−protein folding process semiquantitatively. The strongest driving force for the reaction is the enthalpy associated with the four bonds from zinc(II) to cysteinate and histidine residues, compared with the bonds from zinc(II) to water. The binding of cobalt(II) to CP-1 is less enthalpically driven than the binding of zinc(II) by −7.6 kcal/mol. This value is approximately equal to, but slightly larger than, the expectation based on considerations of ligand field stabilization energy.

The $Cys_2His_2$ zinc finger domain (*1*−*4*) is a widely occurring structural unit found in many eukaryotic gene regulatory proteins. Analysis of the human genome sequence suggests that this domain is the most widely occurring domain encoded in this genome with more than 2500 examples (*5*, *6*). Large numbers of these domains are also encoded in other eukaryotic genomes (*7*, *8*). In the presence of bound zinc (or other appropriate divalent metal ions) these domains adopt a structure consisting of a $\beta$-hairpin followed by a turn and a helix (*2*−*4*). These secondary structural elements are stabilized in the tertiary structure by the coordination of the metal ion between two cysteinate and two histidine residues as well as by a small core involving three relatively conserved hydrophobic residues.

Studies of zinc finger peptides have revealed that these peptides are largely unstructured in the absence of bound metal ions (*4*, *9*−*11*). Essentially, protein folding is fully coupled to metal binding. This stands in contrast to some other metalloproteins, such as the zinc enzyme carbonic anhydrase (*12*) and the blue copper protein azurin (*13*), where the metal-free forms of the protein are completely folded and the metal ion binds to a largely preorganized site. Here, we examine the thermodynamics of the coupled metal binding−protein folding process of a zinc finger peptide through the use of isothermal titration calorimetry. Metal-induced protein folding is an important but relatively understudied chemical process. In addition, the coupled protein folding−metal binding process studied here may be crucial for the functioning of $Cys_2His_2$ domains in gene regulatory proteins that respond to changes in zinc ion concentration (*14*−*16*).

Isothermal titration calorimetry directly measures the heat released during a chemical reaction. Under favorable conditions, titration calorimetry experiments can reveal the stoichiometry, the enthalpy, the free energy, and, hence, the entropy changes that occur over the course of a reaction (*17*, *18*). Furthermore, additional studies can reveal the heat capacity changes as well as providing evidence regarding the uptake or release of protons that occur during the reaction. Our studies have been performed with a prototypical $Cys_2His_2$ zinc finger peptide termed CP-1 (for *c*onsensus *p*eptide *1*). This peptide has the sequence ProTyrLys**Cys**ProGlu**Cys**GlyLysSerPheSerGlnSerSerAspLeuValLys**His**GlnArgThr**His**ThrGly, where the metal binding residues are shown in bold and the conserved hydrophobic residues are underlined. CP-1 and its derivatives have been extensively studied with regard to its metal ion binding affinity and specificity (*10*, *19*, *20*).

## MATERIALS AND METHODS

*Peptide Synthesis and Purification*. Peptides used in this study were synthesized using a Milligen/Biosearch 9050 peptide synthesizer using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry with *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and 1-hydroxy-7-azabenzotriazole (HOAt) activation with or without the modifications of Smith and Berg (*21*). Peptide was cleaved from the resin and deprotected by treatment with reagent K

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[88.5% trifluoroacetic acid (TFA), 5% phenol, 5% water, 2% thioanisole, and 0.5% ethanedithiol] for 3 h. After precipitation in ether, the peptide was washed with cold ether to remove any remaining scavengers. The crude peptide was reduced prior to purification by incubation at room temperature for 1 h in the presence of 2−3 equiv of tris(2-carboxyethylphosphine) hydrochloride (TCEP) per cysteine residue in water. The peptide was purified on a Rainin or Vydac $C_{18}$ reversed-phase HPLC column with an acetonitrile gradient containing 0.1% TFA. Collected fractions were dried under a 95% nitrogen/5% hydrogen atmosphere in a Savant SpeedVac concentrator. All peptide manipulations were performed in this atmosphere to prevent cysteine oxidation. Peptide compositions were confirmed by mass spectrometry.

*Isothermal Titration Calorimetry*. The binding of cobalt(II) and zinc(II) to CP-1 was monitored by isothermal titration calorimetry. The titration experiments were performed on an Omega titration calorimeter (MicroCal, Inc., Northampton, MA.) with a Keithly preamplifier. Measurement of metal binding to a zinc finger peptide requires the maintenance of anaerobic conditions to prevent cysteine oxidation. Therefore, water and buffers for titration experiments were degassed extensively with helium before use and were then stored in an anaerobic chamber. All peptide manipulations were performed in an anaerobic environment. All titration buffers, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), and (2-aminoethyl)trimethylammonium chloride (cholamine), were prepared at 200 mM, pH 7.0, plus 50 mM NaCl. 2-(*N*-Morpholino)ethanesulfonic acid (MES) was prepared at 200 mM plus 50 mM NaCl at pH 6.3, 6.0, and 5.5. All reagents except for cholamine were SigmaUltra reagents. Cholamine was purchased from Aldrich. The pH was adjusted with the highest grade of sodium hydroxide from Fluka. Zinc(II) chloride and cobalt(II) chloride were of the highest available purity from Aldrich. Metal stock solutions were prepared in buffer and then standardized by EDTA titrations, monitored calorimetrically.

For a typical binding experiment, the concentration of the zinc finger peptide (in the syringe) was 0.8−1.5 mM, while the concentrations of metal solution (in the calorimeter cell) ranged from 15 to 30 $\mu$M. Each set of titrations began with a peptide titration into buffer for the purpose of determining dilution effects not directly related to the binding enthalpy. Subsequent experiments incorporated metal in the sample cell solution. A typical titration experiment used 1.7−2.2 nmol of peptide per titration point. This quantity of peptide could be dispensed from the syringe in 1−3 $\mu$L aliquots into the cell.

*Determination of $\Delta H^{\circ}_{ionization}$ for Cholamine Chloride*. The $\Delta H^{\circ}_{\text{ionization}}$ for cholamine chloride was determined experimentally via titration calorimetry, similarly to the method of Fukada and Takahashi (*22*). A dilute solution (2−5 mM) of cholamine chloride was prepared volumetrically in water, with the pH adjusted to 7.4. The cholamine solution was titrated with standardized HCl (Aldrich) until saturation. The data were analyzed similarly to other experimental data.

*Data Analysis*. All data were analyzed using the MicroCal Origin software supplied with the instrument. Before data analysis, the enthalpy of diluting the peptide into buffer only was subtracted from the experimental data. For a typical experiment, the final four points of the titration approximated the heat of dilution within 5%. Each reported measurement is the average of at least three individual experiments.

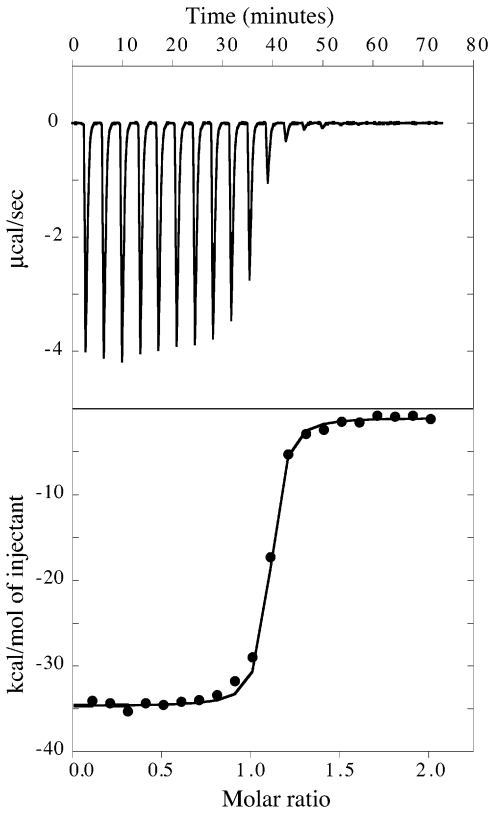


FIGURE 1: Isothermal titration data for the titration of zinc(II) with CP-1 in 200 mM cholamine chloride, pH 7.0, and 50 mM NaCl at 25 °C.

## RESULTS

*Association of CP-1 with Zinc(II) and Cobalt(II)*. The enthalpy of metal binding by CP-1 was directly measured by isothermal titration calorimetry. Figure 1 shows a typical titration profile of the titration for zinc(II) with CP-1 in cholamine buffer at pH 7.0 and 25 °C.

Similar titration profiles were obtained from cobalt(II) binding to CP-1. The binding of zinc(II) to CP-1 is a strongly exothermic reaction. Because previous studies (*10*, *19*) have revealed that the association constant for zinc(II) binding to this peptide is greater than $10^{11}$ $M^{-1}$, the low concentration of reactants necessary to determine accurately the association constant for the reaction cannot be used due to the sensitivity limitations of the instrument. However, an accurate measure of the enthalpy of binding and the binding stoichiometry can be obtained. A summary of all the collected thermodynamic data is shown in Tables 1 [for zinc(II)] and 2 [for cobalt(II)]. For each of the experiments performed, the binding stoichiometry was found to be one peptide per metal ion.

*Protonation Effects on the Binding Enthalpy*. The binding of zinc(II) or cobalt(II) metal to CP-1 is linked to the release of protons from some of the metal binding amino acids and, perhaps, other residues. The number of protons absorbed or released from the peptide during a particular binding reaction can be determined by performing titration experiments in buffers with different ionization enthalpies (*23*). Typically, the observed standard enthalpy change is the sum of two terms:

Table 1: Thermodynamics of the Association of Zinc(II) with CP-1 under Different Temperature, pH, and Buffer Conditions

| pH | buffer | temp (°C) | $K_a$ ($M^{-1}$)[a] | $\Delta H°$ (kcal/mol)[b] |
|---|---|---|---|---|
| 7.0 | cholamine | 25 | $>10^8$ | $-33.7 \pm 0.8$ |
| 7.0 | PIPES | 25 | $>10^8$ | $-23.4 \pm 1.0$ |
| 7.0 | PIPES | 17 | $>10^8$ | $-18.6 \pm 0.8$ |
| 7.0 | PIPES | 34 | $>10^8$ | $-27.3 \pm 0.5$ |
| 7.0 | HEPES | 25 | $>10^8$ | $-27.6 \pm 0.6$ |
| 6.0 | MES | 25 | $>10^8$ | $-19.4 \pm 0.6$ |
| 5.5 | MES | 25 | $>10^8$ | $-15.9 \pm 1.0$ |

[a] Due to the high association constant of the reaction measured, these values were unable to be determined. [b] The enthalpy is the average of at least three measurements. Standard deviations are listed.

Table 2: Thermodynamics of the Association of Cobalt(II) with CP-1 under Different pH Conditions

| pH | buffer | temp (°C) | $K_a$ ($M^{-1}$) | $\Delta H°$ (kcal/mol)[a] |
|---|---|---|---|---|
| 7.0 | PIPES | 25 | $>10^8$ | $-15.9 \pm 2.0$ |
| 6.3 | MES | 25 | $3.0 \times 10^7$ | $-12.8 \pm 0.2$ |
| 6.0 | MES | 25 | $5.1 \times 10^6$ | $-11.7 \pm 1.0$ |
| 5.5 | MES | 25 | $7.8 \times 10^5$ | $-8.3 \pm 0.6$ |

[a] The enthalpy is the average of at least three measurements. Standard deviations are listed.

$$\Delta H°_{obsd} = \Delta H°_{intrinsic} + n\Delta H°_{buffer}$$

where $\Delta H°_{intrinsic}$ is the standard enthalpy of the binding reaction (including enthalpies of protonation or deprotonation of any protein residues), $n$ is the number of protons released over the course of the reaction, and $\Delta H°_{buffer}$ is the standard enthalpy of protonation of the buffer in which the experiment was performed.

Titration experiments were performed in three different buffers: PIPES ($\Delta H°_{buffer} = -2.73$ kcal/mol), HEPES ($\Delta H°_{buffer} = -5.02$ kcal/mol), and cholamine ($\Delta H°_{buffer} = -11.51$ kcal/mol). The number of protons associated with metal binding for CP-1 was estimated from the slope of the plot of the observed enthalpy for the binding reaction in that buffer versus the ionization enthalpy of each buffer. Linear regression analysis yielded $1.1 \pm 0.1$ protons released. This value is lower than the value of 2 expected, assuming that the two cysteine residues are fully protonated in the metal-free form. However, sequence features including the presence of adjacent positively charged residues can shift the p$K_a$ value of cysteine into or below the neutral range (*24*, *25*). The two histidine residues have p$K_a$ values of approximately 6.5 (*10*), indicating that each histidine is expected to release approximately 0.3 proton upon metal binding at pH 7.0.

An alternative method for determining the proton linkage in a binding system is to measure equilibrium constants at different pH values. Titrations of both zinc(II) and cobalt-(II) with CP-1 peptide were performed at pH 7.0, 6.0, and 5.5. Inspection of the zinc titration data (Table 1) shows that the apparent binding constant of CP-1 for zinc(II) is still $>10^8$ $M^{-1}$ at pH 5.5. The cobalt(II) titration data (Table 2 and Figure 2) clearly illustrate the pH dependence of metal binding to CP-1.

However, these data are complicated by the protonation of the histidine residues over the pH range used. Protonation of both histidines in the metal-free form occurs in this range while protonation on the second of the two histidines occurs in the metal-bound form to some extent as well (*10*). The decrease in the observed enthalpy of binding observed for both the zinc(II) and cobalt(II) provides evidence for the occurrence of these processes. Thus, these data provide additional evidence of proton release occurring coupled to the metal binding reaction but are difficult to analyze in quantitative terms. A value of 1.1 protons released during the binding reaction will be used in all subsequent analyses.

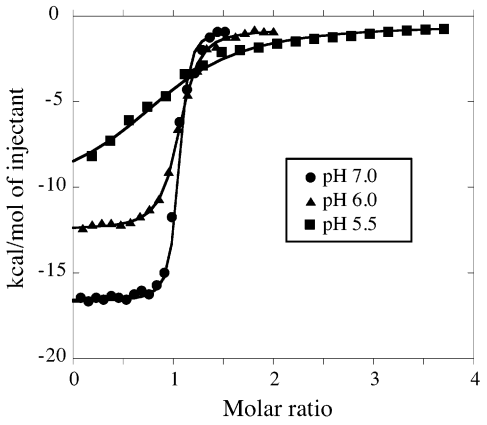


FIGURE 2: Calorimetric data for the titration of cobalt by CP-1 peptide at 25 °C at three different pH values: pH 7.0 in PIPES buffer, pH 6.0 in MES buffer, and pH 5.5 in MES buffer.

The heat capacity change upon metal binding, $\Delta C_p°$, can be determined from titration calorimetry experiments performed at different temperatures. Binding experiments were performed at three temperatures, 17, 25, and 34 °C, under otherwise identical experimental conditions. The $\Delta C_p°$ was determined from the slope of the line generated by plotting the observed enthalpy of the binding reaction versus the temperature at which that experiment was conducted. The heat capacity change for CP-1 binding to zinc(II) was found to be $-514$ ($\pm 18$) cal $K^{-1}$ $mol^{-1}$.

## DISCUSSION

*Experimental Thermodynamic Parameters for Zinc(II) Binding*. A thermodynamic profile of the prototypical zinc finger peptide CP-1 binding to zinc(II) and cobalt(II) has been determined through titration calorimetry. In principle, this analytical method can be used to determine $\Delta H°$, $K_a$, and $n$ for a given chemical reaction. However, for CP-1, the binding constants for zinc(II) and, to a less extent, those for Co(II), are too high to measure calorimetrically at pH 7.0. Nonetheless, since values for these equilibrium constants have been previously determined by other methods (*10*, *19*), a relatively complete thermodynamic analysis is still possible.

In analyzing these data, one must first carefully consider the precise chemical processes that are occurring during the titration experiment. The initial state consists of free peptide which exists in a largely unfolded conformational ensemble. At pH 7.0, both the cysteine residues and the histidine residues are largely in their neutral forms, although some cysteine deprotonation and histidine protonation occurs. The metal ion is in aqueous solution, primarily as hexaaquo ions, but is also potentially interacting with other components of the buffer. In the final state, the CP-1−metal ion complex has formed, and this has a well-defined three-dimensional structure with a small but significant hydrophobic core. Both cysteine residues are deprotonated; the protons released are taken up by the basic component of the buffer. The water

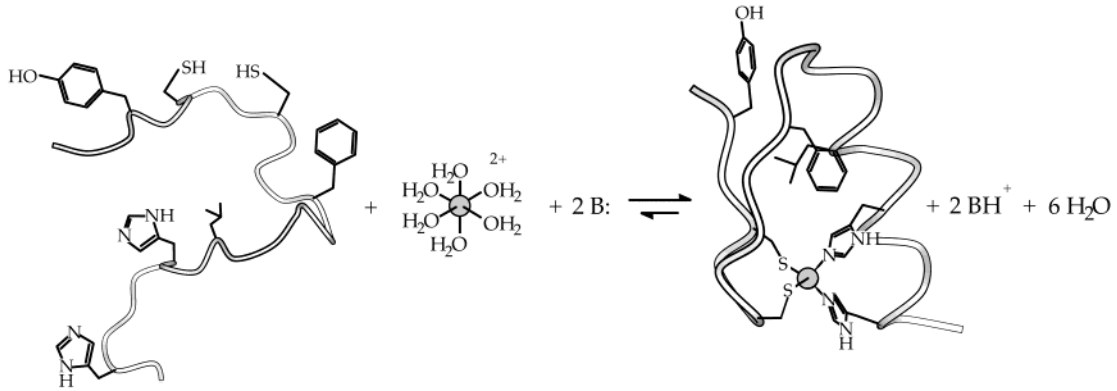


FIGURE 3: Overall process occurring during the titration calorimetry experiment. An unfolded peptide binds a metal ion with the transfer of protons from cysteine to buffer. The release of two protons is shown although the data indicate that only a net of 1.1 protons is released. Six water molecules are released from the metal ion. The metal-bound form of the peptide is folded with the four metal binding residues and the three most conserved hydrophobic residues buried.

molecules that had been coordinated to the metal ion are free in solution. Thus, the overall process is shown in Figure 3.

The experimentally determined enthalpy includes the contribution of proton binding by the buffer. This depends on the identity of the buffer used. We shall focus on the data obtained in cholamine since this buffer has the lowest tendency to bind to metal ions such as zinc(II). Note that the heat of protonation of cholamine is quite large, −11.51 kcal/mol, so that a significant part of the heat released during the reaction (33.7 kcal/mol) is due to buffer protonation. The dissociation constant for the CP-1−zinc(II) complex at pH 7.0 is $5.7 \times 10^{-12}$ M, corresponding to a standard free energy of −15.3 kcal/mol. From these values, the overall standard entropy change for the reaction (including buffer effects) is $\Delta S^\circ_{obs} = (\Delta H^\circ_{obs} - \Delta G^\circ_{obs})/T = -62$ cal mol$^{-1}$ K$^{-1}$.

*Structure-Based Thermodynamic Analysis*. The thermodynamic parameters for the peptide folding component reaction can be estimated from structure-based thermodynamic parameters derived from other protein folding reactions (*26*, *27*). This model assumes that the thermodynamic properties for a protein folding reaction can be deduced from a combination of changes in exposed polar and apolar surface area that are assumed to occur during the process and conformational entropy terms for the peptide backbone and side chains. The parameters used are based on unfolded states modeled in completely extended conformations. On the basis of structures of CP-1−zinc(II) complexes deduced from NMR and crystallographic studies (*28*, *29*), the changes in apolar and polar surface area accompanying the binding reaction are $\Delta A_{apolar} = -789$ Å$^2$ and $\Delta A_{polar} = -565$ Å$^2$. The estimation of thermodynamic terms begins with the calculation of the change in heat capacity associated with folding (*26*). This is given by

$$\Delta C_p^\circ{}_{calc} = 0.45\Delta A_{apolar} - 0.26\Delta A_{polar} = -208 \text{ cal mol}^{-1} \text{ K}^{-1}$$

This is different from the observed value of $\Delta C_p^\circ$ since the experimental value includes contributions to $\Delta C_p^\circ$ not related to the peptide. The folding enthalpy is given by

$$\Delta H^\circ_{calc} = \Delta H^{\circ *}_{calc} + \Delta C_p^\circ{}_{calc}(T - T_H^*)$$

where $\Delta H^{\circ *}_{calc} = 35\Delta A_{polar}$. $\Delta H^{\circ *}_{calc}$ is the polar contribution to the enthalpy at $T = T_H^* = 373$ K, the temperature at which the apolar contribution is assumed to be zero. The second term allows extrapolation from $T_H^*$ to the experimental temperature ($T = 298$ K). For the CP-1−zinc(II) complex, the standard folding enthalpy is calculated to be $\Delta H^\circ_{calc} = -19.8 + 15.6 = -4.2$ kcal/mol.

The entropy change associated with folding is given by

$$\Delta S^\circ_{calc} = \Delta S^{\circ *}_{calc} + \Delta C_p^\circ{}_{calc} \ln(T/T_S^*)$$

where $\Delta S^{\circ *}_{calc}$ is the configurational entropy and the second term involves solvent restructuring extrapolated from $T = T_S^* = 385$ K, the temperature at which the apolar contribution to the entropy is zero. For the CP-1−zinc(II) complex, the standard folding entropy is calculated to be $\Delta S^\circ_{calc} = -123 + 53 = -70$ cal mol$^{-1}$ K$^{-1}$. The configurational entropy is calculated from backbone (*30*) and side chain (*31*) entropy values assuming that the four metal binding residues and the three conserved hydrophobic residues are buried while the remaining residues are exposed. The derived value of −123 cal mol$^{-1}$ K$^{-1}$ is in reasonable agreement with a value of −112 cal mol$^{-1}$ K$^{-1}$ derived from the simpler calculation based on an average change of −4.3 cal mol$^{-1}$ K$^{-1}$ per amino acid for 26 amino acids (*26*). Note that the estimated folding free energy is $\Delta G^\circ_{calc} = \Delta H^\circ_{calc} - T\Delta S^\circ_{calc} = -4.2 - 298(-70)/1000 = +16.7$ kcal/mol, consistent with the fact that this peptide does not fold to a stable structure in the absence of bound metal ions.

Four additional terms must be included. The first involves deprotonation of the cysteine and histidine residues. We determined that 1.1 protons are released during the course of the reaction. On the basis of the p$K_a$ values of the histidne residues, $2 \times 0.3 = 0.6$ proton comes from the histidine residues. We assume that the remaining 0.5 proton is derived from the cysteine residues. Calorimetric data indicate that $\Delta H^\circ = +8.7$ kcal/mol and $\Delta S^\circ = -3$ cal mol$^{-1}$ K$^{-1}$ for histidine deprotonation (*22*) and $\Delta H^\circ = +8.5$ kcal/mol and $\Delta S^\circ = -18$ cal mol$^{-1}$ K$^{-1}$ for cysteine deprotonation (*32*). These terms are compensated for by the corresponding terms for the protonation of the buffer, cholamine, namely, $\Delta H^\circ = -11.5$ kcal/mol and $\Delta S^\circ = +7$ cal mol$^{-1}$ K$^{-1}$. The net result of the protonation terms is $\Delta H^\circ = -3.2$ kcal/mol and $\Delta S^\circ = -3$ cal mol$^{-1}$ K$^{-1}$. The third term involves the entropy associated with the water released from zinc(II) during the reaction. This entropy has been estimated to be 9.5 cal mol$^{-1}$

$K^{-1}$ per water (*33*). Since six waters are released, this corresponds to a standard entropy increase of $\Delta S^\circ = 57$ cal $mol^{-1}$ $K^{-1}$. The final term involves the entropy associated with the reduction of the number of independent particles from two [peptide and $Zn(OH_2)_6^{2+}$] to one [peptide−zinc(II)] complex, given that we have already accounted for water release. This entropy loss is estimated to be −19 cal $mol^{-1}$ $K^{-1}$ on the basis of estimates for the translational, librational, and cratic entropies (*33*). For these considerations, the overall entropy change is estimated to be $\Delta S^\circ_{calc} = -70 + (-3) + 57 + (-19) = -35$ cal $mol^{-1}$ $K^{-1}$. This is in modest agreement with the experimental value of $\Delta S^\circ_{obs} = -62$ cal $mol^{-1}$ $K^{-1}$.

The overall standard enthalpy for the reaction cannot be as readily calculated due to uncertainties about the relative strengths of the six bonds between zinc(II) and water compared with the four bonds between zinc(II) and the cysteinate and histidine ligands. In addition, the effects of other enthalpic interactions such as peptide amide to cysteinate sulfur hydrogen bonds are difficult to estimate. However, a rough analysis is possible. The overall enthalpy change due to protein folding, histidine and cysteine deprotonation, and cholamine protonation is estimated to be $\Delta H^\circ = -4.2 + -3.2 = -7.6$ kcal/mol. The experimental value for the overall enthalpy in cholamine buffer is −33.7 kcal/mol. Thus, the net result of bond breaking to bond forming is a favorable term of $-33.7 - (-7.6) = -26.1$ kcal/mol. Dividing this excess enthalpy equally between the four bonds between zinc(II) and the peptide-derived ligands yields $-26.1/4 = -6.5$ kcal/mol per bond. This value is reasonably consistent with the observed equilibrium constants of $10^3$−$10^4$ $M^{-1}$ (corresponding to free energy changes of −4.1 to −5.4 kcal/mol) observed for *N*-methylimidazole and 2-hydroxyethanethiolate displacement of water from a zinc finger peptide in which the final histidine ligand was not included in the sequence (*35*). Thus, the experimentally observed enthalpy for zinc(II) binding to CP-1 is also consistent with expectations based on parameters related to peptide folding and approximate enthalpies from previously characterized reactions.

*Thermodynamic Preference for Zinc(II) over Cobalt(II).* The binding of cobalt(II) and zinc(II) to CP-1 was each examined in PIPES buffer at pH 7.0 and in MES buffer at pH 6.0 and 5.5. In each case, zinc(II) binding had a more favorable enthalpy with a nearly constant enthalpy difference of −7.6 kcal/mol. Previous studies had revealed that CP-1 binds zinc(II) more favorably than cobalt(II) with a standard free energy difference of $\Delta G^\circ = \Delta G^\circ_{zinc} - \Delta G^\circ_{cobalt} = -16.0 - (-9.8) = -6.2$ kcal/mol (*19*). This difference has been associated with the change in ligand field stabilization energy associated with the transition from an octahedral site in water to a tetrahedral site in the zinc finger peptide, an enthalpic term (*36*). As anticipated, the experimental enthalpy difference nicely accounts for this free energy difference.

*Comparison with Other Systems.* Calorimetric studies of zinc(II) and cobalt(II) binding to apo-carbonic anhydrase have recently been reported (*36*, *37*). This protein differs from CP-1 in two important ways. First, the apo-carbonic anhydrase is essentially completely folded so that metal binding is not coupled to protein folding in the same way it is for CP-1 (*12*). Second, carbonic anhydrase binds the metal ion through only three ligands, all histidines with one or, in some cases, two water molecules remaining bound to the metal center. With zinc(II) in at pH 7.0, the following thermodynamic parameters (corrected for the release of 0.3 proton) were determined:

$$\Delta G^\circ = -16.4 \pm 0.2 \text{ kcal/mol}$$

$$\Delta H^\circ = -6.4 \pm 0.3 \text{ kcal/mol}$$

$$\Delta S^\circ = +34 \pm 0.8 \text{ cal mol}^{-1} \text{ K}^{-1}$$

For comparison, the corresponding values for CP-1 (corrected for proton release) are

$$\Delta G^\circ = -15.3 \pm 0.5 \text{ kcal/mol}$$

$$\Delta H^\circ = -21.1 \pm 1.0 \text{ kcal/mol}$$

$$\Delta S^\circ = -19 \pm 3 \text{ cal mol}^{-1} \text{ K}^{-1}$$

The binding free energy for apo-carbonic anhydrase is quite similar to that for CP-1, but the remainder of the thermodynamic parameters is quite different. Assuming that the metal binding site in carbonic anhydrase is completely preorganized, the binding entropy should be given by the sum of two terms, the entropy of the release of five (of six) waters from the zinc(II) ion and the entropy loss associated with the reduction in the number of particles. Thus, $\Delta S^\circ_{calc} = 5(+9.5 \text{ cal mol}^{-1} \text{ K}^{-1}) + (-19 \text{ cal mol}^{-1} \text{ K}^{-1}) = +29$ cal $mol^{-1}$ $K^{-1}$, in reasonable agreement with the observed value. The large difference in the binding enthalpy values of 15 kcal/mol is striking. This may be due, in part, to the differences between cysteinate and histidine as ligands, but previous studies of variants of CP-1 and carbonic anhydrase have revealed much more modest differences (*10*, *38*). Alternatively, the difference in binding enthalpy may be due to the relative lack of flexibility in the carbonic anhydrase site. The preorganized nature of the site may induce some strain at the zinc(II) center. The preorganized structure may help to dictate a structure that favors catalytic function at the expense of binding enthalpy. The large favorable change in entropy upon binding allows tight binding to occur despite the relatively small favorable binding enthalpy.

Apo-carbonic anhydrase binds cobalt(II) with a standard free energy approximately 6 kcal/mol less favorable than that for zinc(II), a level of specificity for zinc(II) over cobalt(II) quite similar to that for CP-1. However, whereas the specificity for CP-1 is almost entirely enthalpic, a more complex pattern of thermodynamic differences is seen for carbonic anhydrase. For carbonic anhydrase, the binding enthalpy for cobalt(II) is nearly 10 kcal/mol more favorable than that for zinc(II) while the binding entropy is 50 cal $mol^{-1}$ $K^{-1}$ less favorable. These results strongly suggest that the structures of the zinc(II) and cobalt(II) forms of carbonic anhydrase have significantly different structures under the conditions studied, in contrast to the corresponding complexes of CP-1 which appear to have quite similar structures.

## CONCLUSION

A complete thermodynamic analysis of zinc(II) and cobalt(II) binding to the prototypical zinc finger peptide CP-1 has been achieved. The results are consistent with a coupled metal binding−protein folding process. The reactions are driven primarily by enthalpic terms associated with the

stronger bonds formed between the metal ions and the peptide-derived cysteinate and histidine ligands compared with those to water. The substantially unfavorable entropy term associated with peptide folding is very nearly balanced by the favorable entropy associated with water release from the metal ions.

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