Entropy−Enthalpy Compensation in Ionic Interactions Probed in a Zinc Finger Peptide†

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ABSTRACT: Zinc(II) and cobalt(II) binding to a series of zinc finger peptides with different charged residue pairs across from one another in a β-sheet were examined. Previous studies revealed a narrow range of interaction free energies (<0.5 kcal/mol) between these residues. Here, isothermal titration calorimetry studies were performed, revealing a range of over 3 kcal/mol in relative binding enthalpies. Double mutant cycle analysis revealed a range of interaction enthalpies ranging from −3.1 to −3.4 kcal/mol for the Arg−Asp pair to −0.8 kcal/mol for the Lys−Glu pair. The large range of interaction enthalpies coupled with the small range of interaction free energies reveals substantial entropy−enthalpy compensation. The magnitudes of the effects are consistent with the formation of a structurally rigid Arg−Asp contact ion pair but less direct and more mobile interactions involving the other combinations.

The stability of the folded forms of proteins depends on the sum of many relatively weak interactions including ionic interactions, hydrogen bonds, and van der Waals contacts. The free energy is the energetic term that determines the overall impact of a given interaction on stability. Evaluation of the free energy from structural information is complicated since the free energy includes both enthalpic and entropic terms. We have previously investigated the free energies of ionic interactions between two residues in the context of a β-sheet present in a zinc finger peptide (*1*). These peptides appear to be largely unfolded in the absence of bound metal ions but fold to a well-defined structure that includes a small β-sheet, a helix, and several turns. This coupling between metal ion binding and peptide folding for these zinc finger peptides allowed experimentally determined cobalt(II) affinities to be interpreted in terms of folding free energies. Double mutant cycles (*2*) allowed the extraction of interaction free energies between the pair of amino acid residues. These studies revealed that the free energies associated with potential salt bridges were relatively small, less than 0.5 kcal/mol (at pH 7.0 with 50 mM NaCl). These observations were consistent with the free energy ranges observed for solvent-exposed salt bridges in other contexts (*3*−*8*). In addition, we found that interactions involving aspartate led to larger interaction free energies than those involving glutamate.

We have recently reported that isothermal titration calorimetry can be used to monitor metal binding by zinc finger peptides (*9*). The use of calorimetry allows the dissection of binding free energies into enthalpic and entropic components (*10*−*12*), facilitating the analysis of the mechanistic basis for metal binding induced peptide folding and for metal ion binding specificity. Here, we report the use of isothermal titration calorimetry to determine interaction free energies for pairs of oppositely charged residues in the presence of the zinc finger β-sheet. Specifically, studies were performed with peptides based on the consensus zinc finger peptide CP-1 (*13*) with residues substituted into positions 3 and 10 within the sequence ProTyr**Ser**$_3$CysProGluCysGlyLys-**Ser**$_{10}$PheSerGlnLysSerAspLeuValLysHisGlnArg ThrHisThr-Gly where the positions 3 and 10 (shown in bold) correspond to the positions where sequence variation was introduced and the metal binding residues are underlined. A structural representation of the metal-bound form of CP-1 is shown in Figure 1 to indicate the spatial relationship between positions 3 and 10. Peptides with the following pairs of residues were examined: Lys_3Glu_{10}, Lys_3Asp_{10}, Arg_3Glu_{10}, Arg_3Asp_{10},

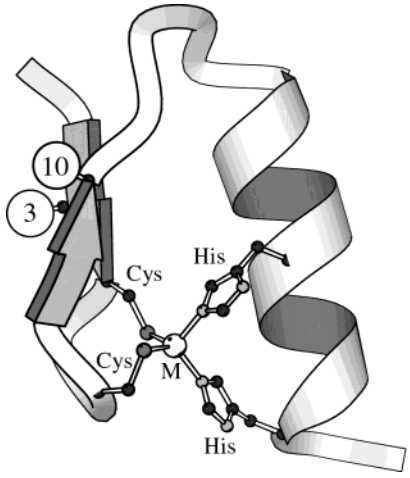

FIGURE 1: Schematic structure (*17*) of a zinc finger domain showing the relationship between the residues in positions 3 and 10 across the β-sheet.

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Glu_3Glu_{10}, and Asp_3Asp_{10}. These studies revealed a much wider range of interaction enthalpies than was found for interaction free energies. These results highlight the importance of entropy−enthalpy compensation (*14*, *15*) in these relatively weak interactions.

MATERIALS AND METHODS

Peptide Synthesis and Purification. All peptides used in this study were synthesized using a Milligen/Biosearch 9050 Peptide Synthesizer using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry with *O*-pentafluorophenyl ester (OPfp) activation. The peptides were cleaved and deprotected from the resin by treatment with Reagent B (88% TFA, 5% phenol, 5% water, and 2% triisopropylsilane) for 2−4 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 incubating at room temperature for 1 h in the presence of 2−3 equiv of 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 identities were confirmed by mass spectrometry.

Isothermal Titration Calorimetry. The binding of cobalt(II) and zinc(II) to the CP-1 variants was monitored by isothermal titration calorimetry as described previously (*9*). 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, chelexed Milli-Q 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. The titration buffer, 200 mM PIPES pH 7.0, 50 mM NaCl, was prepared with SigmaUltra reagents in chelexed Milli-Q water. The pH was adjusted with the sodium hydroxide (99.998%) from Fluka. Zinc(II) chloride (99.999%) and cobalt(II) chloride (99.999%) were of the highest available purity from Aldrich. Metal stock solutions were prepared in buffer and then standardized by EDTA[1] titrations.

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 μM. Each experimental set of titrations began with a peptide titration into buffer for the purpose of determining dilution effects not directly related to the binding reaction. 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 amount of peptide was dispensed from the syringe in 1−3 μL aliquots into the cell.

[1] Abbreviations: TFA, trifluoroacetic acid; TCEP, (Tris[2-carboxyethylphosphine] hydrochloride); PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); NaCl, sodium chloride; EDTA, ethylenediaminetetraacetic acid.

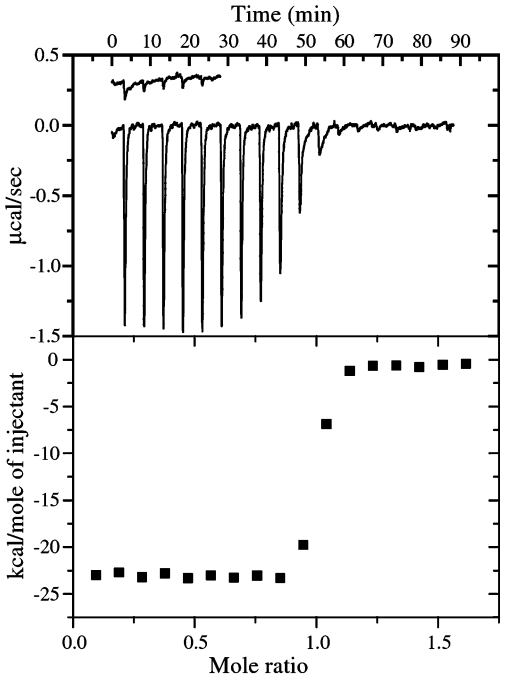

FIGURE 2: Results of a typical titration of a CP-1 variant peptide into zinc(II). The top panel shows the calorimetric scan with an inset showing the result when zinc(II) is not included in the calorimeter cell. The bottom panel shows the results of integration of the above trace, revealing essentially stoichiometric binding.

Table 1: Standard Enthalpies for Metal Ion Binding to the Zinc Finger Peptide CP-1[a]

peptide	$\Delta H°$ (kcal/mol), Zn(II)	$\Delta H°$ (kcal/mol), Co(II)
S_3S_{10}	−22.3 ± 2.0	−14.6 ± 1.5
K_3S_{10}	−22.9 ± 1.1	−14.6 ± 2.1
R_3S_{10}	−22.2 ± 0.3	−14.3 ± 0.5
S_3D_{10}	−20.6 ± 0.2	−12.2 ± 1.0
S_3E_{10}	−21.1 ± 0.3	−13.5 ± 0.8
K_3D_{10}	−22.2 ± 1.1	−13.8 ± 0.3
K_3E_{10}	−22.5 ± 1.1	−14.3 ± 0.2
R_3D_{10}	−23.6 ± 1.1	−15.3 ± 1.6
R_3E_{10}	−22.5 ± 1.3	−14.0 ± 1.4
D_3D_{10}	−20.2 ± 0.3	−12.6 ± 0.4
E_3E_{10}	−22.2 ± 0.7	−13.9 ± 0.5

[a] All values shown are the average of at least three determinations, and estimated standard deviations are shown.

Data Analysis. All data were analyzed with minimal manipulation using the MicroCal Origin software supplied with the instrument. Before data analysis, the enthalpy of diluting the peptide into buffer 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.

RESULTS

Standard enthalpies were determined for zinc(II) and cobalt(II) binding by a series of zinc finger peptides based on the consensus zinc finger peptide CP-1. The results of a typical titration are shown is Figure 2, and the results for all titrations are summarized in Table 1. The ability to use both zinc(II) and cobalt(II) provides validation of the determined enthalpies since a constant difference between the enthalpy values for zinc(II) and cobalt(II) is anticipated over a series

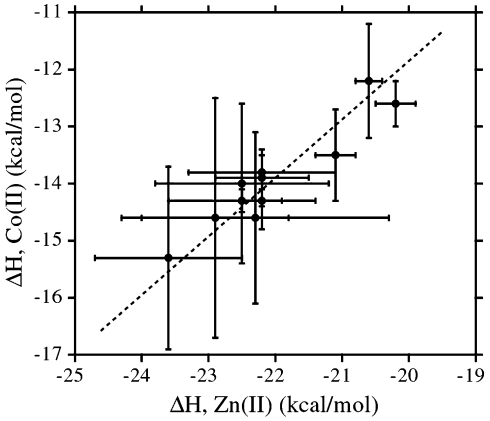

FIGURE 3: Comparison of the enthalpies for zinc(II) binding and cobalt(II) binding to CP-1. The dotted line shown has a slope of 1 and reveals that the enthalpy for zinc(II) binding is 8.1 ± 0.4 kcal/mol more negative that the enthalpy for cobalt(II) binding to the same peptide.

of peptides in which the metal binding site itself is not perturbed. A plot of the enthalpy for cobalt(II) versus the enthalpy for zinc(II) binding is shown in Figure 3. These data can be fit with a line of slope 1 and an offset of 8.1 ± 0.4 kcal/mol ($r = 0.92$). This difference is comparable to that observed previously for the parent peptide CP-1 (*9*).

Calorimetry can often be used to determine binding equilibrium constants in addition to binding enthalpies through curve-fitting methods (*10*−*12*). This requires that sufficient curvature occur in the plot of heat released as a function of peptide added for the determination of an equilibrium binding constant to be determined by curve fitting. A small amount of curvature was sometimes observed in these plots, but this was not reproducible and is due to effects other than equilibrium binding such as underestimation of heat released because of incomplete equilibration.

Double mutant cycles are a useful method to isolate the energetic interactions between two residues of interest (*2*). Four double mutant cycles can be constructed from the peptides that have been studied as shown in Figure 4. For instance, to study the interaction enthalpy between Lys in position 3 and Asp in position 10, the results from four peptides are combined. The reference peptide S_3S_{10} begins the cycle. Mutation of Ser_3 to Lys_3 forms K_3S_{10}, and the enthalpy change associated with this substitution can be calculated by taking the difference between the metal-binding enthalpies for these two peptides. Similarly, the enthalpy change associated with the substitution of Asp for Ser in position 10 can be calculated from the difference between the metal-binding enthalpies for S_3D_{10} and S_3S_{10}. If no significant interaction between Lys in position 3 and Asp in position 10, then the enthalpy difference between the double mutant peptide K_3D_{10} and the reference peptide S_3S_{10} should be equal to the sum of the differences for the two single mutants. The difference between these two values is defined to be the interaction enthalpy, $\Delta\Delta H°_{int}$.

Two additional peptides were studied. These peptides, D_3D_{10} and E_3E_{10}, have negatively charged residues in both positions 3 and 10. In both cases, the metal-binding enthalpies were less favorable for these peptide as compared to the reference peptide S_3S_{10}.

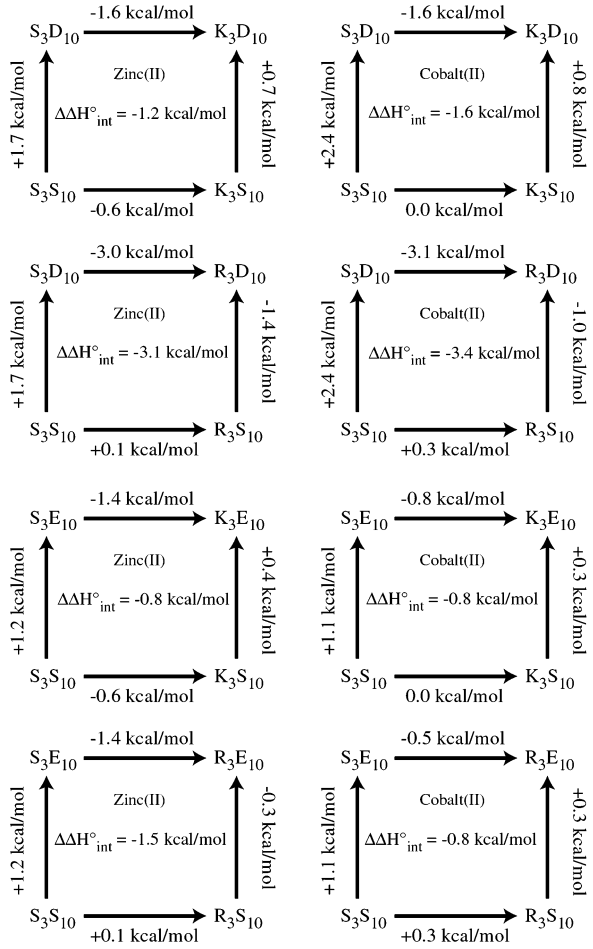

FIGURE 4: Double mutant cycles based on enthalpy changes for both zinc(II) and cobalt(II). The deduced interaction enthalpies are shown.

DISCUSSION

Metal binding enthalpies have been determined for a series of 11 peptides derived from the consensus zinc finger peptide CP-1 using both zinc(II) and cobalt(II). For each metal ion, the binding enthalpies vary over a range of over 3 kcal/mol. This stands in contrast to previous studies of the cobalt(II)-binding free energies where a much smaller range was observed for the same peptides at the same NaCl concentration (*1*). The larger range observed for the enthalpies is crucial for the success of the present studies since the estimated errors in the enthalpy determinations are significantly larger than those from the free energy studies. As noted previously, the good correlation between the results with zinc(II) and cobalt(II) reveals that the differences between the enthalpies that have been determined are significant and should be useful for more detailed analysis. To facilitate this analysis, the results from the zinc(II) and cobalt(II) studies were combined. The interaction enthalpies were determined to be $\Delta\Delta H°_{int}(KD) = -1.3 \pm 1.8$ kcal/mol, $\Delta\Delta H°_{int}(RD) = -3.3 \pm 1.5$ kcal/mol, $\Delta\Delta H°_{int}(KE) = -0.8 \pm 1.8$ kcal/mol, and $\Delta\Delta H°_{int}(RE) = -1.2 \pm 1.4$ kcal/mol. While the estimated standard deviations are relatively large, the interaction enthalpy for the R_3D_{10} peptide is significantly different from that for the other three peptides ($p < 0.10$, student *t*-test).

Table 2: Interaction Free Energies, Interaction Enthalpies, and Deduced Interaction Entropies

	$\Delta\Delta G^\circ_{int}$ (kcal/mol) (from ref *1*)	$\Delta\Delta H^\circ_{int}$ (kcal/mol)	$\Delta\Delta S^\circ_{int}$ (cal/mol K)
KD	−020 ± 0.02	−1.3 ± 1.8	−4 ± 6
RD	−0.11 ± 0.02	−3.3 ± 1.5	−11 ± 5
KE	−0.05 ± 0.02	−0.8 ± 1.8	−3 ± 6
RE	−0.02 ± 0.02	−1.2 ± 1.4	−4 ± 5

The observation that free energies vary only slightly while enthalpies vary over a much larger range indicates that entropy−enthalpy compensation is occurring. Entropy−enthalpy compensation reflects the trade off between forming interactions that are enthalpically favorable at the expense of making the system more rigid. For relatively weak interactions such as the electrostatic interactions that are the subject of the present investigation as well as hydrogen bonds and van der Waals interactions, these terms often compensate for one another nearly exactly (*14*, *15*). On the basis of the previously reported interaction free energies and the determined interaction enthalpies, the interaction entropies can be calculated. The results are $\Delta\Delta S^\circ_{int}(KD) = -4 \pm 6$ cal/mol K, $\Delta\Delta S^\circ_{int}(RD) = -11 \pm 5$ cal/mol K, $\Delta\Delta S^\circ_{int}(KE) = -3 \pm 6$ cal/mol K, and $\Delta\Delta S^\circ_{int}(RE) = -4 \pm 5$ cal/mol K. The interaction free energies, interaction enthalpies, and deduced interaction entropies are summarized in Table 2.

Examination of the interaction enthalpies that have been determined illustrates these effects. The interaction enthalpy between Arg in position 3 and Asp in position 10 was found to be more negative than −3 kcal/mol based on both the zinc(II) and the cobalt(II) data with a combined value of $\Delta\Delta H^\circ_{int}(RD) = -3.3 \pm 1.5$ kcal/mol. The interaction free energy for these residues was found to be $\Delta\Delta G^\circ_{int}(RD) = 0.11 \pm 0.02$ kcal/mol at the same salt concentration. Thus, the interaction entropy is $\Delta\Delta S^\circ_{int}(RD) = +11 \pm 5$ cal/mol K. In contrast, for Lys in position 3 and Asp in position 10, the interaction enthalpy is substantially smaller in magnitude, $\Delta\Delta H^\circ_{int}(KD) = -1.3 \pm 1.8$ kcal/mol. The interaction free energy, however, is slightly more favorable, $\Delta\Delta G^\circ_{int}(KD) = -0.20 \pm 0.02$ kcal/mol so that the interaction entropy is relatively small, $\Delta\Delta S^\circ_{int}(KD) = +4 \pm 6$ cal/mol K. One explanation that rationalizes these observations involves the conformational entropies associated with the side chains involved. Computational studies have allowed estimation of the conformational entropies for side chains in solvent exposed positions such as those under study (*16*). For the Arg−Asp pair, the conformation entropy lost if both side chains go from the unconstrained environment assumed for the calculations to a single, fixed conformation would be −9.1 cal/mol K. This is quite similar to the deduced interaction entropy between these two residues of −11 ± 5 cal/mol K. Thus, our results are consistent with the formation of a true contact ion pair between Arg and Asp, perhaps stabilized by the electrostatic interaction as well as two hydrogen bonds as shown in Figure 5. These interactions would be responsible for the relatively large, negative interaction enthalpy observed. Modeling studies indicate that these interactions lock these side chains in relatively fixed positions, leading to the compensating entropy change. The net result is the modest interaction free energy that is observed.

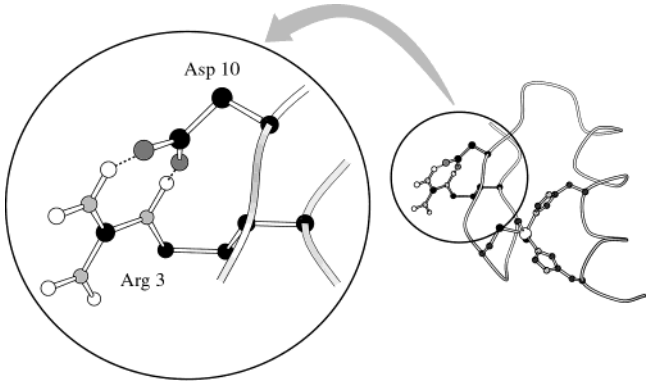

FIGURE 5: Schematic structure showing a potential contact ion pair between the side chains of Arg in position 3 and Asp in position 10. Forming two hydrogen bonds between these side chains should be enthalpically favorable but entropically unfavorable as these interactions substantially reduce the conformational possibilities for both side chains.

For the Lys−Asp pair, the observed interaction entropy is $\Delta\Delta S^\circ_{int}(KD) = -4 \pm 6$ cal/mol K. This interaction entropy appears to be smaller than the entropy change estimated if both the Lys and the Asp were locked into single conformations ($\Delta S^\circ = -7.9$ cal/mol K), although the relatively large standard deviation in the experimental value softens this conclusion. This observation as well as the lower interaction enthalpy suggests that these two side chains do not form a rigid ion pair but, instead, interact in a less intimate way. Similar analyses apply to the Lys−Glu and Arg−Glu pairs where the calculated conformational entropy losses upon adopting single conformations are 9.4 and 10.6 cal/mol K, respectively.

For the peptides with negatively charged residues in both positions, impact of the inclusion of these residues on the enthalpy is larger than the impact on free energy, particularly for the Asp−Asp pair. This indicates that entropic stabilization is able to partially compensate for the enthalpic destabilization associated with two residues with charges of the same sign held in relatively close proximity in the folded form of the peptide.

CONCLUSION

Previous studies with these peptides had revealed that the interaction free energies between pairs of residues placed in positions within a β-sheet such that they have the potential to form salt bridges were quite modest. From these values, it was not possible to determine if the residues did, indeed, form contact ion pairs but with counteracting desolvation energies leading to small free energy effects or if the residues were only interacting through longer range electrostatic interactions. Through the use of isothermal titration calorimetry and double mutant cycle analysis, significant differences between pairs of residues that show similar interaction free energies have been observed. These observations reinforce the notion that relatively subtle structural effects can lead to significantly different detailed thermodynamic behavior. Entropy−enthalpy compensation nearly completely masks these effects when observed free energy alone. The results suggest further investigations of the differences in dynamic behavior of the side chains in these peptides through NMR or other suitable methods.

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