Isothermal Titration Calorimetry Measurements of Ni(II) and Cu(II) Binding to His, GlyGlyHis, HisGlyHis, and Bovine Serum Albumin: A Critical Evaluation

Yi Zhang, Shreeram Akilesh, and Dean E. Wilcox*

Department of Chemistry, Dartmouth College, Hanover, New Hampshire 03755-3564

Received January 6, 2000

The binding of Ni(II) and Cu(II) to histidine, to the tripeptides GlyGlyHis and HisGlyHis, and to the protein bovine serum albumin has been studied by isothermal titration calorimetry (ITC) to determine the experimental conditions and data analysis necessary to reproduce literature values for the binding constants and thermodynamic parameters. From analysis of the ITC data, we find that there are two major considerations for the use of this method to accurately quantify metal ion interaction with biological macromolecules. First, to determine true pH-independent binding constants, ITC data must be corrected for metal ion competition with protons by accounting for the experimental pH and pK_a values of the metal-binding residues. Second, metal interaction with the buffer (stability and enthalpy of formation of metal−buffer complex(es)) must be included in the analysis of the ITC data to determine the binding constants and the change in enthalpy. While it may be possible to use a buffer that forms only weak, and therefore negligible, complexes with the metal, a buffer that has a strong and well-characterized interaction has the benefit of suppressing metal ion hydrolysis and precipitation, and of allowing the quantification of high-affinity metal-binding sites on biological macromolecules. This study has also quantified the contribution of the N-terminal imidazole of HisGlyHis to the stability of the Cu(II) and Ni(II) complexes of this protein sequence and has provided new insight about Cu(II) binding to albumin.

Introduction

Calorimeters have now been developed that are capable of accurately and reproducibly measuring the heat flow associated with the interaction of biological macromolecules in dilute aqueous solution.[1,2] In particular, isothermal titration calorimetry (ITC) is being used with increasing frequency because of its potential to provide detailed thermodynamic, and even kinetic, information about these binding events.

Since quantitative measurements of the interaction of metal ions with biological macromolecules are important for understanding their physiological roles and pathological effects, ITC has considerable potential for elucidating the biochemistry of metal ions. In fact, two of the earliest studies using a new sensitive ITC instrument characterized Fe(III) binding to ovotransferrin[3] and human transferrin.[4] However, this method has not yet been used extensively to quantify metal interaction with proteins, DNA, or RNA.

As part of our studies of the coordination chemistry of peptides and proteins that are rich in His and/or Cys, we have begun using ITC to quantify the thermodynamics of metal binding. However, it soon became apparent that there are several potential complications for studies involving transition-metal ions. While some of these have been mentioned previously,[4,5] our attempts to reproduce binding constants and enthalpy changes in the literature have identified a number of factors that must be considered. Here we report the results of a critical evaluation of ITC measurements of Ni(II) and Cu(II) binding to the amino acid His, to the tripeptides GlyGlyHis and HisGlyHis, and to the protein bovine serum albumin (BSA). From this study we have developed guidelines for ITC experimental parameters and data analysis to accurately quantify the thermodynamics of metal binding to biological macromolecules.

Materials and Methods

All reagents were obtained from Sigma Chemical Co. and were ≥99% pure. Nickel, copper, and histidine stock solutions (100 mM) were made by dissolving $NiSO_4 \cdot 6(H_2O)$, $CuSO_4 \cdot 5(H_2O)$, and histidine in Nanopure (>18 M Ω resistance) water. Phosphate, HEPES (*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)), TES (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid), and Tris (tris-(hydroxymethyl)aminomethane) buffer solutions were made by dissolving equimolar amounts of acid and base forms of the buffer salt in Nanopure water, and adjusting to the desired pH with HCl or NaOH. Experimental solutions were made by diluting stock solutions into the buffer solutions and resulted in negligible dilution of the buffer. The tripeptide GlyGlyHis (GGH) and bovine serum albumin (BSA) were purchased from Sigma and used without further purification. The tripeptide HisGlyHis (HGH) was synthesized from Fmoc-protected amino acids and standard solid-phase peptide synthesis procedures at the bench,[6] and purified by reversed-phase HPLC. The peptide was characterized by its ^{1}H NMR spectrum, and its purity was determined by analytical HPLC to be 95%.

ITC measurements were carried out at 25 (± 0.2) °C on a MicroCal OMEGA ultrasensitive titration calorimeter. The titrant and sample solutions were made from the same stock buffer solution, and both

* To whom correspondence should be addressed. Phone: (603) 646-2874. Fax: (603) 646-3946. E-mail: dean.e.wilcox@dartmouth.edu

(1) Wiseman, T.; Williston, S.; Brandts, J. F.; Lin, L.-N. *Anal. Biochem.* **1989**, *179*, 131−137.
(2) Freire, E.; Mayorga, O. L.; Straume, M. *Anal. Chem.* **1990**, *62*, 950A−959A.
(3) Lin, L.-N.; Mason, A. B.; Woodworth, R. C.; Brandts, J. F. *Biochemistry* **1991**, *30*, 11660−11668.
(4) Lin, L.-N.; Mason, A. B.; Woodworth, R. C.; Brandts, J. F. *Biochemistry* **1993**, *32*, 9398−9406.
(5) McLendon, G.; Hull, H.; Larkin, K.; Chang, W. *J. Biol. Inorg. Chem.* **1999**, *4*, 171−174.
(6) Private communication with Fernando Albericio.

10.1021/ic000036s CCC: $19.00
Published on Web 06/07/2000

experimental solutions were thoroughly degassed before each titration. The solution in the cell was stirred at 800 rpm by the syringe to ensure rapid mixing. Typically, 8−10 μL of titrant was delivered over 25 s with an adequate interval (6−20 min) between injections to allow complete equilibration. Titrations continued until 4−5 equiv had been added to ensure that no additional complexes were formed in excess titrant. A background titration, consisting of the identical titrant solution but only the buffer solution in the sample cell, was subtracted from each experimental titration to account for heat of dilution. Figures 1−3 show the raw ITC data on top, while the bottom of each figure shows a plot of heat flow per mole of titrant versus the molar ratio of titrant to the species in the sample cell for each injection, after subtraction of the background titration.

The data were collected automatically and subsequently analyzed with either a one-site or two-site binding model by the Windows-based Origin software package supplied by MicroCal. The Origin software uses a nonlinear least-squares algorithm (minimization of χ^2) and the concentrations of the titrant and the sample to fit the heat flow per injection to an equilibrium binding equation, providing best fit values of the stoichiometry (n), change in enthalpy ($\Delta H°$), and binding constant (K).

Results and Analysis

Histidine. ITC measurements of Ni(II) and Cu(II) binding to the amino acid His were made to assess the ability of this method to reproduce the reported binding constants and enthalpy changes. Histidine is a tridentate ligand for these metal ions,[7] binding through its carboxylate ($pK_a = 1.7$), imidazole ($pK_a = 6.0$), and amine ($pK_a = 9.1$). Both Ni(II) and Cu(II) bind to His in 1:1 and 1:2 complexes, characterized by the following equilibria in the pH range 6.5−8.5:

$$M^{2+} + His \rightleftarrows M(His)^+ + H^+ \quad (1)$$

$$M(His)^+ + His \rightleftarrows M(His)_2 + H^+ \quad (2)$$

Comparison of titrations of the metal ion into a solution of His ($M^{2+} \rightarrow$ His) and titrations of His into a solution of the metal ion (His $\rightarrow M^{2+}$) showed that both equilibria could be determined more accurately by fitting data from the latter experiment.

Figure 1 shows the ITC data for a His $\rightarrow$ Ni(II) titration in 100 mM HEPES buffer at pH 7.6. Evidence is observed for each binding equilibrium (inflection at ~1 and ~2 molar equivalents of His), both of which are net exothermic, and an excellent fit of these data is achieved for a model with two independent binding sites and all six fit parameters floating freely (Table 1, fit 1). However, this best fit indicates that the stoichiometry of each equilibrium deviates significantly from the expected value of 1.0. This could be due to error(s) in solution concentrations but, since these are sequential binding equilibria, an inaccurate concentration of titrant or sample would result in deviation of both n_1 and n_2 in the same direction from 1.0. To further evaluate this point, these data were fit with the constraint that $n_1 = n_2$ (Table 1, fit 2). While this gives the more reasonable stoichiometry of 1.09, both quantitatively (χ^2) and qualitatively (fit 2 of Figure 1) this resulted in a somewhat poorer fit, as would be expected with fewer independently adjustable parameters.

As noted elsewhere[8] and found in the analysis of these and other ITC data in this study, certain parameters of the two-site fit are not well defined when $K_1 \neq K_2$ and both binding events

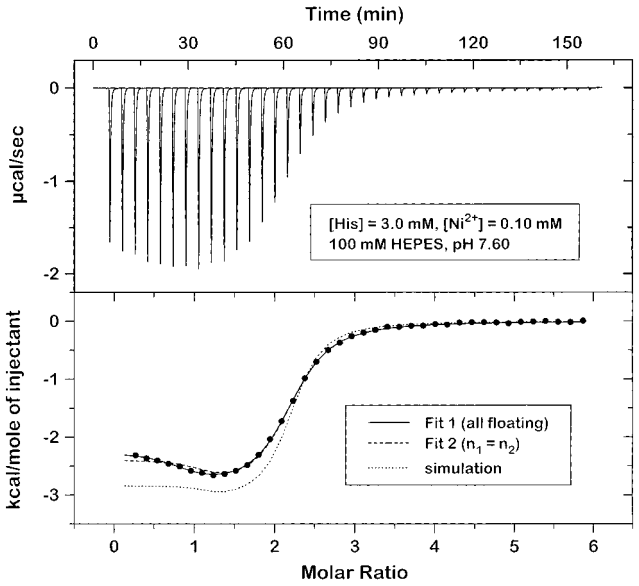

Figure 1. ITC titration of 0.10 mM Ni(II) with 3.0 mM His in 100 mM HEPES buffer at pH 7.60. Parameters for fit 1, fit 2, and simulation are found in Table 1.

have the same sign of $\Delta H°$. In particular, a relatively large error is associated with the value of $K_{1,ITC}$ and, depending on the relative magnitudes of K_1 and K_2, reaction stoichiometry becomes a soft fit parameter. Thus, deviation of the stoichiometries from 1.0 appears to be largely an artifact of fitting these data to the two-site model. However, since the stoichiometry of both binding equilibria (eqs 1 and 2) are known, it is chemically reasonable to fix $n_1 = n_2$, which we have done for all reported fits of ITC data for Ni(II) and Cu(II) binding to His.[9]

The reported stability constants for $Ni(His)^+$ and $Ni(His)_2$ have been obtained by potentiometric titrations and are pH-independent values.[10] Thus, our experimental values at pH 7.60 ($K_{1,ITC}$, $K_{2,ITC}$) need to be corrected for deprotonation of the amine upon Ni(II) binding by the following relationships:

$$K_1 = \frac{[NiHis^+]}{[Ni^{2+}][His^-]} = \frac{[NiHis^+]}{[Ni^{2+}]}\frac{[H^+] \times 10^{9.09}}{[His]} = K_{1,ITC} \times 10^{9.09-pH} \quad (3)$$

$$K_2 = \frac{[NiHis_2]}{[NiHis^+][His^-]} = \frac{[NiHis_2]}{[NiHis^+]}\frac{[H^+] \times 10^{9.09}}{[His]} = K_{2,ITC} \times 10^{9.09-pH} \quad (4)$$

In addition, a minor correction is also required to account for the small percentage of His with protonated imidazole at this pH. As seen for 100 mM HEPES buffer (Table 2), log K_1 is 5.2% lower than its literature value, while the difference between log K_2 and its literature value is only 1.7%.[11]

Two pieces of evidence suggest that there is an interaction between Ni(II) and the HEPES buffer that leads to the low value of K_1, although this interaction is weak and has not been quantified. First, there is a discrepancy between the experimental

(7) Kiss, T. In *Biocoordination Chemistry: Coordination Equilibria in Biologically Active Systems*; Burger, K., Ed.; Ellis Horwood: New York, 1990; pp 91−95 and references therein.
(8) ITC Data Analysis in Origin, MicroCal Inc., Northampton, MA, 1993.
(9) Certain data sets, in fact, could not be fit with all six parameters freely floating and required the constraint that $n_1 = n_2$.
(10) Martell, A. E., Smith, R. M., Simeon, V. I., Eds. *Critical Stability Constants*; Plenum: New York, 1989.
(11) Quantitative comparison of binding constants are generally made between log K values.

Table 1. Best Fit and Simulation Parameters of the ITC Data in Figure 1 to a Two-Site Model

	fit 1 (all floating)	fit 2 ($n_1 = n_2$)	simulation[b]
n_1	0.83 (± 0.03)	n_2	1.09
$K_{1,ITC}$	3.7×10^6 ($\pm 1.2 \times 10^6$)	4.9×10^6 ($\pm 1.6 \times 10^6$)	1.8×10^7
$\Delta H°_{1,ITC}$[a]	−2.25 (±0.04)	−2.39 (±0.02)	−2.84
n_2	1.35 (±0.03)	1.09 (±0.003)	1.09
$K_{2,ITC}$	2.06×10^5 ($\pm 0.08 \times 10^5$)	1.67×10^5 ($\pm 0.09 \times 10^5$)	2.85×10^5
$\Delta H°_{2,ITC}$[a]	−2.97 (±0.04)	−3.00 (±0.06)	−3.14
χ^2	207	995	

[a] kcal/mol. [b] See the text for the source of simulation parameters.

Table 2. Best Fit Parameters of the His → Ni(II) and His → Cu(II) ITC Data to a Two-Site Model (Indicated by the ITC Subscript)[a]

buffer (pH)	$n_{1,ITC}$	log $K_{1,ITC}$	$\Delta H°_{1,ITC}$[b] ($\Delta H°_{1,calcd}$)[b,c]	log K_1	$n_{2,ITC}$	log $K_{2,ITC}$	$\Delta H°_{2,ITC}$[b] ($\Delta H°_{2,calcd}$)[b,c]	log K_2	log(K_1K_2)
A. Ni(II)									
100 mM HEPES (7.60)	$n_{2,ITC}$	6.692	−2.39 (−2.84)	8.211	1.09	5.223	−3.0 (−3.14)	6.742	14.953
100 mM Tris (8.10)	$n_{2,ITC}$	6.675	−6.16 (−8.91)	8.467	1.08	4.821	−5.80 (−9.21)	7.189	15.656
25 mM phosphate (6.80, $I = 0.1$ M)	$n_{2,ITC}$	5.886	1.50 (2.09)	8.662	1.13	4.588	1.02 (1.79)	6.946	15.608
20 mM HEPES (7.50, $I = 0.1$ M)	$n_{2,ITC}$	6.193	−2.16 (−2.79)	7.808	1.04	5.053	−3.00 (−3.09)	6.668	14.476
25 mM Tris (8.23, $I = 0.1$ M)	$n_{2,ITC}$	6.378	−6.99 (−8.86)	7.744	0.917	5.270	−6.66 (−9.16)	7.023	14.767
reported values[d]				8.66				6.86	15.52
B. Cu(II)									
20 mM Tris (8.10)	1.05	5.793	−3.95 (−11.81)	9.364[e]	$n_{1,ITC}$	4.278	0.865 (−10.22)	8.914[e]	18.268
				7.868[f]				10.414[f]	(18.282)
100 mM Tris (8.10)	1.06	5.033	−4.10 (−11.81)	9.975[e]	$n_{1,ITC}$	2.258	0.940 (−10.23)	8.292[e]	18.267
				7.761[f]				10.491[f]	(18.252)
reported values[d]				10.16				7.91	18.07

[a] K_1 and K_2 are the pH-independent binding constants calculated from $K_{1,ITC}$ and $K_{2,ITC}$, which also account for Ni(II) and Cu(II) interaction with the buffer in the case of Tris and phosphate. [b] kcal/mol. [c] Change in enthalpy calculated from enthalpy changes for His deprotonation, metal-His binding, and buffer protonation. [d] Reference 10. [e] Assumes two Tris ligands are replaced by one His for each step. [f] Assumes one Tris ligand is replaced by the first His and three Tris ligands are replaced by the second His.

$\Delta H°_{1,ITC}$ value and a calculated value ($\Delta H°_{1,calcd}$), which assumes contributions to the net enthalpy change from *only* deprotonation of the amine (and the small percentage of protonated imidazole), Ni(II) binding to His^-, and protonation of the buffer (Table 2). A more exothermic reaction is predicted, thereby implicating an endothermic dissociation of Ni(II) from HEPES. Second, in the early part of the titration, when His would be displacing weakly bound HEPES, there is a difference between the experimental ITC data and a simulated set of ITC data (Figure 1), which was generated from stoichiometries of the $n_1 = n_2$ fit, the $\Delta H°_{1,calcd}$ and $\Delta H°_{2,calcd}$ values, and the literature K_1 and K_2 values, corrected for deprotonation of the amine and the imidazole at this pH (Table 1).

Identical His → Ni(II) ITC measurements in Tris buffer at pH 8.10 were analyzed for proton displacement upon Ni(II) binding, as indicated above, but the resulting values of K_1 and K_2 (log $K_1 = 7.665$; log $K_2 = 5.811$) were significantly lower than their literature values, indicating a Ni−buffer interaction that competes with Ni(II) binding to His.[12] This is also indicated by $\Delta H°_{1,calcd}$ and $\Delta H°_{2,calcd}$ values that are significantly more negative than the $\Delta H°_{1,ITC}$ and $\Delta H°_{2,ITC}$ values, respectively. Nickel−Tris complexes have been characterized,[10] and this interaction was subsequently included in the analysis of the His → Ni(II) ITC data in Tris buffer.

In 100 mM Tris at pH 8.10, Ni(II) in the sample cell is found predominantly as $Ni(Tris)_2^{2+}$ (82%) and $Ni(Tris)^{2+}$ (17%). Assuming that the predominant equilibria involved in the His titration under these conditions are

$$Ni(Tris)_2^{2+} + His \rightleftarrows Ni(Tris)(His)^+ + HTris^+ \quad (5)$$

$$Ni(Tris)^{2+} + His + Tris \rightleftarrows Ni(Tris)(His)^+ + HTris^+ \quad (6)$$

$$Ni(Tris)(His)^+ + His \rightleftarrows Ni(His)_2 + HTris^+ \quad (7)$$

stability constants for $Ni(Tris)_2^{2+}$ (log $\beta_2 = 4.6$) and $Ni(Tris)^{2+}$ (log $\beta_1 = 2.63$) were used to account for Ni−Tris interactions in eqs 5−7, which were then used in calculations of K_1 and K_2. The resulting log K values (Table 2) are 2.2% lower and 4.8% higher than the reported values, respectively, suggesting that these assumptions are reasonable but do not completely account for the displacement of Tris by His. The major contribution to these discrepancies probably originates from the mixed ligand and buffer complexes of unknown stability in eqs 5−7. Another factor may be the reported slow kinetics of Ni−Tris binding,[13] although a sufficient delay was made between injections to allow the heat flow to return to the baseline. The log product of K_1 and K_2 differs from the reported product by 0.9%, indicating that this analysis of the 100 mM Tris ITC data does a reasonably good job of accounting for the overall equilibrium involving formation of the $Ni(His)_2$ species.[14]

We have also obtained His → Ni(II) ITC measurements with lower buffer concentrations and added salt to maintain constant ionic strength, and have analyzed these data as described above (Table 2). The experimental data and best fit for a measurement in 25 mM phosphate buffer at pH 6.8 and 50 mM KNO_3 are shown in Figure 2. Surprisingly both equilibria now are net endothermic because of the much smaller protonation enthalpy of HPO_4^{2-} ($\Delta H°_{prot} = -1.1$ kcal/mol), relative to that of HEPES ($\Delta H°_{prot} = -5.1$ kcal/mol) and other Good buffers.[15] Accounting

(12) This interaction is not subtracted by the control titration that accounts for heat of dilution.

(13) Bologni, L.; Sabatini, A.; Vacca, A. *Inorg. Chim. Acta* **1983**, *69*, 71−75.

(14) A similar analysis was undertaken of ITC measurements in 100 mM TES buffer at pH 7.60; however, Ni−TES complexes are not well characterized, thus preventing a complete analysis of these ITC data.

(15) Christensen, J. J., Hansen, L. D., Izatt, R. M., Eds. *Handbook of Proton Ionization Heats and Related Thermodynamic Quantities*; Wiley: New York, 1976.

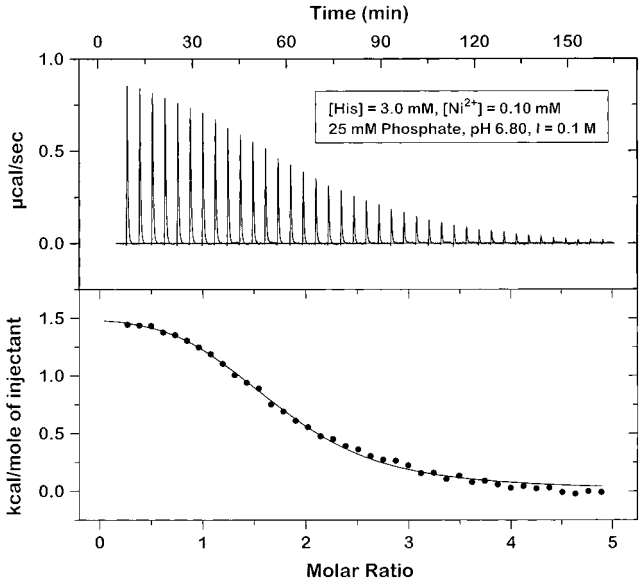

Figure 2. ITC titration of 0.10 mM Ni(II) with 3.0 mM His in 25 mM phosphate buffer at pH 6.80 with 50 mM KNO_3.

for Ni(II) interaction with the phosphate buffer ions,[16] in addition to proton competition at this pH, results in values of log K_1, log K_2, and log K_1K_2 that differ from their literature values by <0.1%, 1.3%, and 0.6%, respectively.

For ITC measurements in 20 mM HEPES and 25 mM Tris, analysis of the ITC data results in values that are lower than those found at 100 mM buffer concentration (Table 2). In the case of the weakly coordinating HEPES, it is possible that hydroxo ligand contributions at this lower buffer concentration affect binding of the first His, resulting in the lower K_1 value. This also may explain the lower K_1 value in 25 mM Tris at pH 8.23. However, another factor in the case of Tris, where we account for Ni−buffer interaction, is a different distribution of the initial Ni(II) species (40% $Ni(Tris)^{2+}$ and 54% $Ni(Tris)_2^{2+}$) under these conditions and the lack of stability data for mixed Ni(II) complexes of Tris and His, as indicated above. These factors have significantly less effect on the K_2 values, which are close to the literature value. In fact, the log K_2 value in HEPES at two different conditions of buffer concentration, pH, and ionic strength differs by only 1.1%. A somewhat larger difference (2.4%) is found in Tris, where different buffer concentrations result in different percentages of mixed Ni−ligand−buffer complexes that are not accurately included in the data analysis.

The above analysis of His → Ni(II) ITC data indicates that accurate binding constants can be obtained when the contribution of metal−buffer species to the overall equilibrium can be quantitatively subtracted, which is most easily achieved when a single well-characterized metal-buffer complex is present. This insight then guided His → Cu(II) ITC measurements in 20 and 100 mM Tris buffer at pH 8.10 (Table 2).[17] Under both conditions, the predominant species in the sample cell initially is $Cu(Tris)_4^{2+}$ (log $\beta_4 = 14.1$),[10] and the titrant His is expected to displace the buffer in a stepwise fashion. Although this results in several equilibria, we considered two simple cases where either one or two buffer ligands are displaced by the first His. The latter model gives K_1 and K_2 values that are closer to the literature values, while the former gives K_1 and K_2 values that are very similar for both 20 and 100 mM Tris, which is expected because $Cu(Tris)_4^{2+}$ is the predominant species at both buffer concentrations. Since the literature values were determined by unbuffered potentiometric titrations and the analysis of our ITC data necessarily includes mixed Cu−Tris−His species, we do not expect our individual K_1 and K_2 values to match the literature values, but we do expect this of our K_1K_2 product. Further support for the model where the first His displaces only one Tris ligand is found in a comparison of $\Delta H°_{1,ITC}$ and $\Delta H°_{2,ITC}$, which should be similar if each His displaces two Tris ligands but, in fact, are not; the slightly endothermic value of $\Delta H°_{2,ITC}$ is consistent with the greater endothermic contribution from loss of three Tris in the second step. However, choice of a model for the mechanism of His displacement of Tris does not have a significant effect on the overall equilibrium (K_1K_2) for the formation of $Cu(His)_2$, which is very similar for both models at both Tris concentrations. We find that our best value for the log stability constant of $Cu(His)_2$ differs by 1.0% from the literature value, indicating that the overall equilibrium is determined relatively well with this analysis of ITC data obtained under these conditions.

Table 3 contains the experimental ITC thermodynamic data obtained for Ni(II) and Cu(II) binding to His in 100 mM Tris buffer at pH 8.10 and a detailed thermodynamic comparison involving literature values for relevant equilibria. This analysis of both the experimental and literature data quantitatively includes contributions from the different His protonation species at this pH and the relevant metal−Tris complexes at this buffer concentration. The $\Delta G°$ values obtained from the ITC data are in excellent agreement (1.1% and 8.8% difference for Ni(II) and Cu(II), respectively) with values determined from relevant literature equilibrium values, indicating that we are accurately accounting for all significant thermodynamic contributions in the ITC experiment. This analysis was also used to determine $\Delta H°$ and $\Delta S°$ for the species $Ni(Tris)_2^{2+}$, $Ni(Tris)^{2+}$ and $Cu(Tris)_4^{2+}$, which were important for analysis of subsequent ITC data obtained in Tris buffer.

Tripeptides. The above results guided the analysis of our ITC data for Cu(II) and Ni(II) binding to GGH, for comparison to literature values, and to the related peptide HGH. At pH > 6.0 for Cu(II) and pH > 7.5 for Ni(II), each metal ion binds to GGH in a 1:1 complex with square planar coordination through the N-terminal amine ($pK_a = 8.06$), the two deprotonated amides, and the imidazole of the His.[18] However, because a 1:2 complex can form under conditions of excess GGH, the binding data were obtained as tripeptide → M^{2+} ITC titrations. Relative to the His → M^{2+} ITC measurements, these titrations took longer to reach equilibrium, particularly at aliquots approaching 1:1 stoichiometry. Table 4 shows the best fit parameters for these titrations in 100 mM Tris at pH 9.1. The value $K_{XGH,exptl}$ indicates the eq 8 pH-dependent metal binding constant of the peptide (X = G, H), after accounting for metal−buffer interaction.

$$M^{2+} + XGH^{-1} \rightleftarrows M(XGH)^{-} + 2H^{+} \qquad (8)$$

The log binding constant for Cu(II) that we determine from ITC measurements differs from the most recent literature value[19]

(16) For $Ni(HPO_4)$, log $K = 2.1$, and for $Ni(H_2PO_4)^+$, log $K = 0.5$; under these experimental conditions 60% and <2% of the Ni(II) is initially in these two complexes, respectively.

(17) We did not use HEPES because of its reported redox reaction with Cu(II) (Hegetschweiler, K.; Saltman, P. *Inorg. Chem.* **1986**, *25*, 107−109).

(18) Sovago, I. In *Biocoordination Chemistry: Coordination Equilibria in Biologically Active Systems*; Burger, K., Ed.; Ellis Horwood: New York, 1990; pp 162−164 and references therein.

(19) Hay, R. W.; Hassan, M. M.; Chen, Y.-Q. *J. Inorg. Biochem.* **1993**, *52*, 17−25.

Table 3. Thermodynamic Parameters for Ni(II) and Cu(II) Binding to His at pH 8.10 Obtained from ITC Measurements in 100 mM Tris Buffer at 25 °C[a]

reactions	log K	$\Delta G°$ [b]	$\Delta H°$ [b]	$\Delta S°$ [c]
A. Ni(II) ($x = 0.82$, $y = 0.90$, $z = 0.0075$)				
overall ITC experiment equilibrium				
$x\mathrm{Ni(Tris)_2^{2+}} + (1-x)\mathrm{Ni(Tris)^{2+}} + 2y\mathrm{His} + 2z\mathrm{His^+} + 2(1-y-z)\mathrm{His^-} \rightleftarrows \mathrm{Ni(His)_2} + 2(y+2z)\mathrm{TrisH^+} + (1+x-2y-4z)\mathrm{Tris}$	9.63[d]	−13.15	−11.96	3.99
literature equilibria[e]				
$2y(\mathrm{His} \rightleftarrows \mathrm{His^-} + \mathrm{H^+}$	−9.09	12.41	10.50	−6.41)
$2z(\mathrm{His^+} \rightleftarrows \mathrm{His^-} + 2\mathrm{H^+}$	−15.11	20.63	17.50	−10.51)
$x(\mathrm{Ni(Tris)_2^{2+}} \rightleftarrows \mathrm{Ni^{2+}} + 2\mathrm{Tris}$	−4.60	6.28	**6.77**	**1.64**[f])
$(1-x)(\mathrm{Ni(Tris)^{2+}} \rightleftarrows \mathrm{Ni^{2+}} + \mathrm{Tris}$	−2.63	3.59	**3.42**	**−0.57**[f])
$1(\mathrm{Ni^{2+}} + 2\mathrm{His^-} \rightleftarrows \mathrm{Ni(His)_2}$	15.54	−21.22	−16.50	15.83)
$2(y+2z)\ (\mathrm{H^+} + \mathrm{Tris} \rightleftarrows \mathrm{TrisH^+}$	8.10	−11.06	−11.36	−1.01)
	9.53	**−13.01**		
B. Cu(II) ($x = 0.98$, $y = 0.90$, $z = 0.0075$)				
overall ITC experiment equilibrium				
$x\mathrm{Cu(Tris)_4^{2+}} + (1-x)\mathrm{Cu(Tris)_3^{2+}} + 2y\mathrm{His} + 2z\mathrm{His^+} + 2(1-y-z)\mathrm{His^-} \rightleftarrows \mathrm{Cu(His)_2} + 2(y+2z)\mathrm{TrisH^+} + (4-2y-4z)\mathrm{Tris}$	2.46[d]	−3.36	−3.16	0.67
literature equilibria[e]				
$2y(\mathrm{His} \rightleftarrows \mathrm{His^-} + \mathrm{H^+}$	−9.09	12.41	10.50	−6.41)
$2z(\mathrm{His^+} \rightleftarrows \mathrm{His^-} + 2\mathrm{H^+}$	−15.11	20.63	17.50	−10.51)
$x(\mathrm{Cu(Tris)_4^{2+}} \rightleftarrows \mathrm{Cu^{2+}} + 4\mathrm{Tris}$	−14.10	19.25	**18.87**	**−1.30**[f])
$(1-x)(\mathrm{Cu(Tris)_3^{2+}} \rightleftarrows \mathrm{Cu^{2+}} + 3\mathrm{Tris}$	−11.10	15.16	*g*	*g*
$1(\mathrm{Cu^{2+}} + 2\mathrm{His^-} \rightleftarrows \mathrm{Cu(His)_2}$	18.07	−24.67	−20.40	14.34)
$2(y+2z)\ (\mathrm{H^+} + \mathrm{Tris} \rightleftarrows \mathrm{TrisH^+}$	8.10	−11.06	−11.36	−1.01)
	2.26	**−3.09**		

[a] Parameters in bold are derived from a thermodynamic cycle consisting of measured values. [b] kcal/mol. [c] cal/(mol·K). [d] $K = K_{1,\mathrm{ITC}}K_{2,\mathrm{ITC}}\ f(x,y,z)$, where $f(x,y,z)$ is a function that accounts for the relative contributions of different His protonation species and metal−Tris complexes to the overall equilibrium. [e] Reference 10. [f] Derived from $\Delta G° = \Delta H° - T\Delta S°$. [g] Negligible contribution.

Table 4. Best Fit Parameters for ITC Measurements of Cu(II) and Ni(II) Binding to Peptides GlyGlyHis and HisGlyHis in 100 mM Tris at pH 9.1 and 25 °C

	n_{ITC}	log K_{ITC}	$\Delta H°_{\mathrm{ITC}}$[a]	log $K_{\mathrm{XGH,\ exptl}}$	log $K_{\mathrm{XGH,rpt}}$
A. GlyGlyHis[b]					
Cu(II)	0.95	6.615	−5.45	−1.16	−1.550[c]
Ni(II)	0.85	7.188	−7.53	−8.417	−6.932[c]
B. HisGlyHis[d]					
Cu(II)	1.13	6.954	−4.93	−1.277	
Ni(II)	1.01	7.417	−7.94	−8.188	

[a] kcal/mol. [b] 1.10 mM GlyGlyHis; 0.080 mM M^{2+}. [c] Reference 19. [d] 0.40 mM HisGlyHis; 0.050 mM M^{2+}.

by 4.3%. In the case of Ni(II), however, our K value at pH 9.1 is 2 orders of magnitude smaller than the literature value. Because Ni(II) forms complexes with Tris that are not as stable as those of Cu(II), it is likely that there are Ni−hydroxide species at this pH that affect this value of K_{GGH} determined by ITC. This is supported by GGH → Ni(II) ITC titration data at pH 8.0 that give, after appropriate analysis of protonation species and Ni−Tris complexes, log $K_{\mathrm{GGH,exptl}} = -7.226$, which differs by only 4.2% from the literature value.[20]

Table 5 contains the experimental ITC thermodynamic data obtained for Cu(II) and Ni(II) binding to GGH in 100 mM Tris buffer at pH 9.1 and literature thermodynamic values for relevant equilibria, including $\Delta H°$ and $\Delta S°$ values for the species $\mathrm{Cu(Tris)_4^{2+}}$, $\mathrm{Ni(Tris)_2^{2+}}$, and $\mathrm{Ni(Tris)^{2+}}$ determined above. In contrast to Table 3, here the experimental ITC values are used to determine the thermodynamic parameters associated with the eq 8 equilibrium for metal binding to GGH. The thermodynamics of Cu(II) binding to GGH have been studied previously by ITC measurements of pH titrations.[21] Although the reported eq 8 binding constant determined by this method (log $K = -0.48$) is an order of magnitude larger than the more recent potentiometric titration value (log $K = -1.550$)[19] and our ITC value (log $K = -1.616$), there is reasonably good agreement between the previous calorimetry measurement of the enthalpy change for the eq 8 equilibrium ($\Delta H° = -1.25$ kcal/mol) and our value ($\Delta H° = -1.852$ kcal/mol).

Since our ITC measurements of Cu(II) and Ni(II) binding to GGH and HGH were obtained under identical conditions, a direct comparison of the best fit parameters allows us to determine the thermodynamic contribution of the imidazole side chain on the N-terminal residue. The data in Table 4 indicate that both Cu(II) and Ni(II) bind more tightly to HGH than to GGH, and this higher stability quantitatively corresponds to an additional change in free energy of 0.46 and 0.31 kcal/mol for Cu(II) and Ni(II), respectively.

Bovine Serum Albumin. Since our ultimate objective is to use ITC to quantify the interaction of metal ions with biological macromolecules, we extended this study to include Cu(II) binding to BSA. These experiments used metal → protein ITC titrations and incorporated insight gained from the above studies to factor out metal−buffer interaction. Since this protein has an N-terminal sequence of AspThrHis- (DTH-) and both Cu(II) and Ni(II) bind at this sequence in a structure similar to that found with GGH,[22] we expected similar data.

Figure 3 shows the Cu(II) → BSA ITC data and three fits of these data. These data clearly indicate a more complicated interaction of Cu(II) with this protein than expected, with an initial rapid net endothermic binding followed by a slower net exothermic binding up to a 1:1 molar ratio. Thereafter only the

(20) GGH → Cu(II) titration at pH 8.0 gives log $K_{\mathrm{GGH,exptl}} = -0.583$; we suspect that the difference between this and the pH 9.1 value indicates that there are minor thermodynamic contributions from mixed Cu−GGH−Tris species, which we are unable to consider in our analysis.

(21) Arena, G.; Rizzarelli, E.; Sarkar, B. *Inorg. Chim. Acta* **1979**, *37*, L555−L557.

(22) Harford, C.; Sarkar, B. *Acc. Chem. Res.* **1997**, *30*, 123−130 and references therein.

Table 5. Thermodynamic Parameters for Cu(II) and Ni(II) Binding to GlyGlyHis at pH 9.07 Obtained from ITC Measurements in 100 mM Tris Buffer at 25 °C[a]

reactions	log K	$\Delta G°$ [b]	$\Delta H°$ [b]	$\Delta S°$ [c]
A. Cu(II) ($y = 0.92$)				
overall ITC experiment equilibrium				
$Cu(Tris)_4^{2+} + yGGH^- + (1-y)GGH \rightleftarrows CuGGH^- + (3-y)TrisH^+ + (1+y)Tris$	0.49[d]	−0.67	−6.62	−19.94
literature equilibria[e]				
$(1-y)(GGH \rightleftarrows GGH^- + H^+$	−8.02	12.41	*f*	*f*)
$1(Cu(Tris)_4^{2+} \rightleftarrows Cu^{2+} + 4Tris$	−14.10	19.25	***18.87***	***−1.30***)
$1(Cu^{2+} + GGH^- \rightleftarrows CuGGH^- + 2H^+$	**−1.62**	**2.21**	**−1.85**	**−16.55**[g])
$(3-y)(H^+ + Tris \rightleftarrows TrisH^+$	8.10	−11.06	−11.36	−1.01)
B. Ni(II) ($x = 0.89$, $y = 0.92$)				
overall ITC experiment equilibrium				
$xNi(Tris)_2^{2+} + (1-x)Ni(Tris)^{2+} + yGGH^- + (1-y)GGH \rightleftarrows NiGGH^- + (3-y)TrisH^+ + (x+y-2)Tris$	3.47[d]	−4.73	−7.53	−9.40
literature equilibria[e]				
$(1-y)(GGH \rightleftarrows GGH^- + H^+$	−8.02	12.41	*f*	*f*)
$x(Ni(Tris)_2^{2+} \rightleftarrows Ni^{2+} + 2Tris$	−4.60	6.28	***6.77***	***1.64***)
$(1-x)(Ni(Tris)^{2+} \rightleftarrows Ni^{2+} + Tris$	−2.63	3.59	***3.42***	***−0.57***)
$1(Ni^{2+} + GGH^- \rightleftarrows NiGGH^- + 2H^+$	**−8.42**	**11.50**	**9.70**	**−6.03**[g])
$(3-y)(H^+ + Tris \rightleftarrows TrisH^+$	8.10	−11.06	−11.36	−1.01)

[a] Parameters in bold are derived from a thermodynamic cycle consisting of measured values and values derived from Table 3 (bold and italic). [b] kcal/mol. [c] cal/mol·K. [d] $K = K_{1,ITC}K_{2,ITC}\,f(x,y,z)$, where $f(x,y,z)$ is a function that accounts for the relative contributions of different GGH protonation species and metal−Tris complexes to the overall equilibrium. [e] Reference 10. [f] Negligible and/or unknown contribution. [g] Derived from $\Delta G° = \Delta H° - T\Delta S°$.

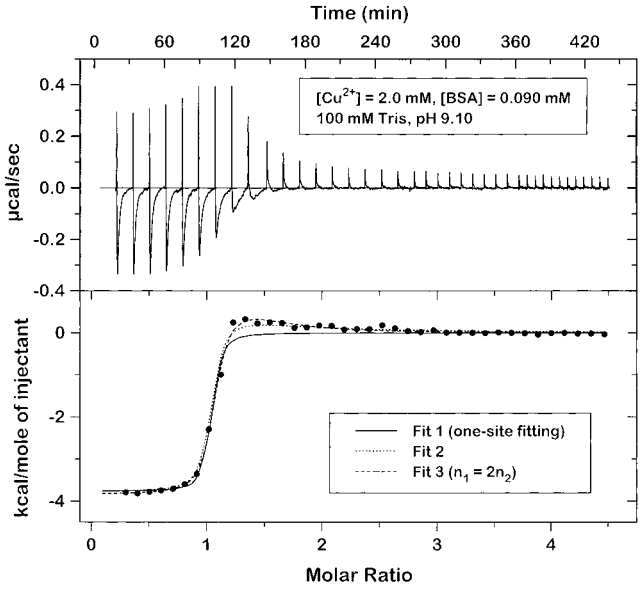

Figure 3. ITC titration of 0.090 mM bovine serum albumin with 2.0 mM Cu(II) in 100 mM Tris buffer at pH 9.10. Parameters for all three fits of the data are found in Table 5.

rapid net endothermic binding remains for another ~0.5 equiv of metal ion. Since the thermodynamic product is Cu(II) bound to the N-terminal site and Cu(II) binds to GGH under similar conditions with a net exothermic enthalpy change, we attribute the slower exothermic process to Cu(II) binding to the N-terminal residues of BSA. This protein contains a single free thiol, Cys34, that constitutes the site where Zn(II), Fe(II), Cd(II), and other metal ions bind,[23] and we suggest that the rapid net endothermic event involves Cu(II) binding initially to this site (kinetic intermediate), prior to migration to the N-terminal site. After a 1:1 stoichiometry of Cu(II) is achieved at the N-terminal site, the ITC data suggest that additional Cu(II) binds more weakly to the Cys34 site.[24] In support of this interpretation, preliminary ITC measurements of Cu(II) binding to BSA that has its Cys34 blocked by reaction with *N*-ethylmaleimide lack the rapid endothermic binding and give Cu(II) binding parameters that are similar to those found with GGH.

Table 6 contains the results from fitting these data, which was not possible with a two-site model and all parameters freely floating. We began with a fit to a one-site model (fit 1) to provide fixed parameters for the first site of a two-site model (fit 2). This fit indicated that the stoichiometry of the weaker site was ~0.5 and allowed the final fit 3 with the fixed relationship $n_1 = 2n_2$.

Copper(II) binding to BSA has been quantified by ultrafiltration[25] and equilibrium dialysis[26] methods, and the stability constant for the 1:1 complex has been reported to be 1.6×10^{13} (pH 7.5) and 1.1×10^{12} (pH 7.4), respectively. Accounting for the experimental pH and protonation of the N-terminal amine ($pK_a = 7.73$), the eq 8 binding constants for these two studies are 4.62×10^{-2} and 6.01×10^{-3}, respectively, which are similar to our ITC result of 2.78×10^{-2}.[27]

Since Cu(II) has a similar coordination when bound to GGH and to the N-terminal DTH- sequence of BSA, and our ITC measurements were obtained under identical conditions, direct comparison indicates that Cu(II) binds somewhat more tightly to BSA ($K = 1.06 \times 10^7$) than to GGH ($K = 4.12 \times 10^6$). However, this difference is considerably less when the pK_a of

(23) Peter, T., Jr. *All About Albumin. Biochemistry, Genetics, and Medical Applications*; Academic Press: San Diego, 1996; see also references therein.

(24) It has been reported (Kratochwil, N. A.; Ivanov, A. I.; Patriarca, M.; Parkinson, J. A.; Gouldsworthy, A. M.; Murdoch, P. D. S.; Sadler, P. J. *J. Am. Chem. Soc.* **1999**, *121*, 8193−8203) that Cys34 may not be fully reduced in commercially available samples of albumin and the ~0.5 stoichiometry for the second Cu(II) may reflect partial oxidation of this thiol in our BSA sample.

(25) Giroux, E.; Schoun, J. *J. Inorg. Biochem.* **1981**, *14*, 359−362.

(26) Ryall, R. G., Ph.D. Thesis, Australian National University, 1974, as cited in ref 25.

(27) An equilibrium dialysis study of Cu(II) binding to BSA (Masuoka, J.; Jegenauer, J.; Van Dyke, B. R.; Saltman, P. *J. Biol. Chem.* **1993** *268*, 21533-21537) reports an intrinsic (pH-independent) binding constant for the 1:1 complex of 1.3×10^{11}; however, it is not clear whether deprotonation of the two amides of the N-terminal DTH-sequence upon Cu(II) binding was included in the analysis, and the report of a Scatchard plot for Cu(II) binding at pH 5.0 that is identical to those at pH 7.0 and 8.5 is at odds with potentiometric and spectroscopic data for Cu(II) binding to a number of XXH- sequences.

Table 6. Best Fit Parameters of the ITC Data in Figure 3

	fit 1 (one-site model)	fit 2[a]	fit 3[b]
n_1	1.00 (± 0.01)	1.00	$2n_2$
$K_{1,ITC}$	6.4×10^6 ($\pm 2.2 \times 10^6$)	6.4×10^6	1.06×10^7 ($\pm 0.27 \times 10^7$)
$\Delta H°_{1,ITC}$[c]	−3.76 (±0.07)	−3.76	−3.84 (± 0.03)
n_2		0.52 (±0.20)	0.51 (±0.002)
$K_{2,ITC}$		1.51×10^4 ($\pm 0.67 \times 10^4$)	6.3×10^4 ($\pm 2.3 \times 10^4$)
$\Delta H°_{2,ITC}$[c]		0.96 (±0.28)	0.96 (±0.10)
χ^2	24002	10067	963

[a] Two-site model with the first set of parameters fixed at values obtained from fit 1. [b] Two-site model with $n_1 = 2n_2$. [c] kcal/mol.

the N-terminal amine is considered; the corresponding eq 8 binding constants are 2.78×10^{-2} and 2.42×10^{-2}, respectively. These results also indicate that the enthalpy change for Cu(II) binding to the N-terminal site of BSA ($\Delta H°_{ITC} = -3.84$ kcal/mol) is less exothermic than it is for Cu(II) binding to GGH ($\Delta H°_{ITC} = -5.45$ kcal/mol).

Discussion

Since heat flow accompanies all binding events, isothermal titration calorimetry has the potential to quantify the interaction of biological molecules with any metal ion. In previous studies, ITC has been used to characterize Fe(III) binding to ovotransferrin[3] and human transferrin,[4] Mg(II) binding to ribonuclease H,[28] Mg(II) and Mn(II) binding to the Klenow fragment of DNA polumerase I,[29] transition-metal binding to concanavalin A,[30] Zn(II) binding to a zinc finger peptide of the HIV nucleocapsid protein,[5] and Ca(II) or Mg(II) binding to a number of proteins and oligonucleotides. However, to the best of our knowledge, there has been no study that has assessed the accuracy and problems associated with ITC measurements of metal ion binding to biologically relevant ligands. Such a critical evaluation is important because only a portion of the total heat flow measured by ITC may be associated with the metal−ligand interaction of interest. Specifically, binding of displaced proton(s) by the buffer will give an exothermic contribution, but release of metal ions from complexes with the buffer will give an endothermic contribution. While contributions such as these may not be relevant for the interaction of two biological macromolecules, thermodynamic parameters obtained from a fit of ITC data need to be critically analyzed whenever metal ions are involved.

We have undertaken an evaluation of ITC measurements of Ni(II) and Cu(II) binding to an amino acid, small peptides, and a protein to uncover potential complications to achieving accurate thermodynamic values. Our primary goal was to determine the experimental conditions and the data analysis that were necessary to reproduce binding constants and thermodynamic parameters that had been obtained with other methods, and thereby outline a protocol for ITC measurements of unknown systems. However, new insight has also been obtained for metal binding to XXH- peptide sequences and for Cu(II) binding to BSA. Below we identify and discuss several key points about ITC measurements of metal binding to proteins and peptides.

While ITC measurements can, in theory, be done in opposite directions (exchanging the species in the sample cell and in the syringe), both practical and interpretive reasons need to be considered. For example, Ni(II) → His ITC titrations involve the sequential equilibria,

$$Ni^{2+} + 2His \rightleftarrows Ni(His)_2 + 2H^+ \quad (9)$$

$$Ni^{2+} + Ni(His)_2 \rightleftarrows 2Ni(His)^+ \quad (10)$$

where $K_9 = K_1K_2$ and $K_{10} = K_1/K_2$ relate the equilibrium constants for these equations to those for eqs 1 and 2. As expected, we find that the best fit parameters determined for the His → Ni(II) ITC titration data in Figure 1 give an excellent fit to reverse Ni(II) → His ITC titration data, using the above relationships between the equilibrium constants. The eqs 9 and 10 equilibria have stoichiometries of 0.5 and 1.0, respectively, but, depending on the relative magnitude of K_1 and K_2, the eq 10 equilibrium may not be evident in the ITC data, thus preventing quantification of the eqs 1 and 2 equilibria. Our His → Ni(II) ITC data have clear evidence for both equilibria and give best fit values that correspond to literature values for the 1:1 and 1:2 complexes. In this case only His → Ni(II) ITC measurements lead to accurate results. For practical reasons (e.g., solubility and availability), the biological macromolecule typically will be in the sample cell, and fitting M^{n+} → macromolecule ITC data can give thermodynamic information about individual metal-binding sites on the macromolecule.

Noninteger stoichiometries were an initial concern. Some variablity in these values can originate from inaccuracy of the concentration(s) of the sample solutions, but in this study it was primarily an artifact of fitting the data to more than one binding site with different metal affinities. This will be of concern when more than one metal ion binds to a biological macromolecule, and independent measurement of the metal-to-macromolecule stoichiometry may be helpful when ITC data are fitted for cases that involve multiple metal-binding sites.

Metal ion displacement of protons is a major consideration in ITC measurements. Comparison of the binding constants obtained from our His → M^{2+} ITC data to binding constants determined from potentiometric titrations of metal−His solutions required knowledge of the amine and imidazole pK_a values. However, relevant literature for metal binding to GGH reports the pH-dependent eq 8 stability constant, and it was not necessary to include the pK_a values associated with the two amide protons that are displaced by Cu(II) or Ni(II) for this comparison. Clearly, for cases where metal ions compete with protons, the number of protons and the associated pK_a values must be known for accurate determination of intrinsic pH-independent binding constants. We have found that analysis of ITC data is more straightforward if an experimental pH can be chosen that results in a single dominant protonation species, as is the case for His in the pH range 6.8−8.1.

It is essential to know the number of protons that are displaced by the metal ion to be able to extract the enthalpy of metal binding from ITC data because buffer protonation enthalpy can be a significant contribution to the total heat flow. This can be clearly seen by comparing Figures 1 and 2, where the difference in protonation enthalpy of the two buffers reverses the sign of the total change in enthalpy. ITC measurements with different

(28) Huang, H.-W.; Cowan, J. A. *Eur. J. Biochem.* **1994**, *219*, 253−260.
(29) Black, C. B.; Cowan, J. A. *J. Biol. Inorg. Chem.* **1998**, *3*, 292−299.
(30) Schwarz, F. P. *J. Inorg. Biochem.* **1993**, *52*, 1−16.

buffers and/or at different pH values can be used to determine the number of protons involoved in binding. In one case, the difference in the total enthalpy change between two ITC measurements with different buffers at the same pH was associated with the difference in protonation enthalpy of the two buffers and was used to estimate the number of displaced protons.[4] A more general approach compares ITC data with different buffers at the same pH and ITC data with the same buffer at different pH values.[31,32] However, the use of different buffers to determine proton contributions to the enthalpy change can pose additional problems when metals are involved, as indicated below.

A particularly important factor in ITC measurements involving metal ions is the contribution of metal−buffer interaction to the binding thermodynamics. Because of the need to maintain constant pH, buffer concentrations are typically high enough that their complexes with metal ions can be the predominant metal species in solution. This introduces competing equilibria, with associated enthalpy changes that contribute to the total heat flow measured by the calorimeter. This additional enthalpy is not subtracted from the control titration, which only accounts for the heat of dilution of the concentrated species in the syringe.

Buffer complexes of metal ions can provide certain experimental advantages for ITC. This complexation can enhance metal ion solubility, particularly at higher pH where metal−hydroxo species begin to form. In addition, depending on the stability of the metal−buffer complex, the buffer can serve as a competing ligand, thereby allowing the measurement of binding constants that are too large ($K > 10^8$) to be measured directly by ITC. The competing ligand nitrilotriacetic acid (NTA) was used for this purpose in studies of Fe(III) binding to ovotransferrin[3] and human transferrin.[4] However, the advantage of using a buffer with sufficiently high metal ion affinity for this purpose is that its concentration remains essentially constant throughout the titration, whereas the concentration of a chelating ligand in the sample cell is constantly changing during the course of a M(chelate)$^{n+}$ → macromolecule ITC titration. As with the choice of an experimental pH to ensure a single dominant protonation species, it is advantageous to choose experimental conditions that favor a single well-characterized metal−buffer species in solution. However, both the stability and the enthalpy of this complex need to be known to account for its contribution to the overall thermodynamics of metal ion interaction with the biological macromolecule.

In addition to critically evaluating ITC measurements involving metal ions, this study also quantitatively compared the thermodynamics of Cu(II) and Ni(II) binding to GGH and HGH to determine the metal binding contribution of an imidazole ligand at the N-terminus of an XXH tripeptide. Since ITC data for metal binding to both tripeptides were obtained under identical conditions, the best fit K_{ITC} and $\Delta H°_{ITC}$ values can be compared directly to determine the contribution(s) of the N-terminal His. In each case the additional imidazole results in a more stable metal−tripeptide complex ($\Delta\Delta G°_{Cu} = -0.46$ kcal/mol; $\Delta\Delta G°_{Ni} = -0.31$ kcal/mol). However, for Cu−HGH this is due to entropic factors ($\Delta\Delta H°_{Cu} = +0.51$ kcal/mol; $\Delta\Delta S°_{Cu} = +3.3$ cal/mol·K), while enthalpic factors dominate in the case of Ni−HGH ($\Delta\Delta H°_{Ni} = -0.41$ kcal/mol; $\Delta\Delta S°_{Ni} = -0.3$ cal/mol·K). This would be consistent with imidazole displacement of an axial aqua ligand in the case of Cu−HGH, but suggests a stronger axial bonding interaction between Ni(II) and the imidazole in Ni−HGH.[33]

Finally, our ITC results on Cu(II) binding to BSA are intriguing, as they suggest a kinetic intermediate, which is associated with a second weaker Cu(II)-binding site. The affinity of BSA for a second Cu(II) has been reported ($K = 5.2 \times 10^6$),[34] but correlation with our ITC value, which is 2 orders of magnitude smaller, will require additional characterization of this Cu(II)-binding site. Preliminary ITC measurements of Ni(II) binding to BSA lack the rapid net endothermic binding event but do indicate that more than one metal ion binds to the protein. The binding site occupied by the first equivalent of Ni(II), however, has an enthalpy change that is similar in sign and magnitude to that found for Ni(II) binding to GGH, suggesting that the most stable Ni(II)-binding site is the N-terminus of BSA. Additional calorimetric and spectroscopic studies of Cu(II) and Ni(II) interaction with this protein are currently in progress.

Summary

An ITC study of Cu(II) and Ni(II) binding to His, GGH, and BSA was undertaken to evaluate the experimental conditions and data analysis necessary to reproduce literature values for the binding constants and thermodynamics of the interaction of these metal ions and biological ligands. This has led to guidelines for ITC measurements of metal binding to biological macromolecules based on the following points.

(1) When possible, the analysis of metal competition with protons should be simplified by choosing a pH that gives a single dominant protonation species of the biological ligands that bind the metal ion.

(2) Choose a buffer whose interaction with the metal ion (stoichiometry, stability, and enthalpy of the metal−buffer complex) is known and conditions that result in a single dominant metal−buffer species in solution. While a buffer with negligible affinity for the metal may be preferred, thermodynamics of the metal−buffer interaction can be subtracted, and a strong metal−buffer interaction may allow the characterization of high-affinity metal-binding sites.

(3) In cases of multiple metal-binding sites, stoichiometries obtained from fitting ITC data may show significant deviations from the reaction stoichiometry, depending on the relative magnitude of the binding constants and enthalpy changes.

(4) Complete thermodynamic analysis requires knowledge of the number of protons involved and the relevant pK_a values and protonation enthalpies. Determination of pH-independent binding constants requires the pK_a values of metal-binding ligands on the biological macromolecule, while quantification of the thermodynamics of metal binding also requires ligand and buffer protonation enthalpies and the formation enthalpies of metal−buffer species.

Acknowledgment. We are grateful to Fernando Albericio for help with the peptide synthesis. The MicroCal OMEGA calorimeter was purchased with funds from the Howard Hughes Medical Institute to Dartmouth College.

IC000036S

(31) Doyle, M. L.; Louie, G.; Monte, P. R. D.; Sokoloski, T. D. *Methods Enzymol.* **1995**, *259*, 183−194.
(32) Baker, B. M.; Murphy, K. P. *Biophy. J.* **1996**, *71*, 2049−2055.
(33) Optical data indicate a small difference in the Ni(II) ligand field of Ni−HGH, relative to that of Ni−GGH.
(34) Zgirski, A.; Frieden, E. *J. Inorg. Biochem.* **1990**, *39*, 137−148.