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Nuclear Magnetic Resonance Studies of the Solution Chemistry of Metal Complexes. 21. The Complexation of Zinc by Glycylhistidine and Alanylhistidine Peptides

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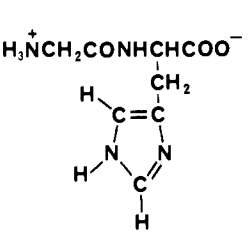
Abstract: The binding of zinc by the peptides glycyl-L-histidine and L-alanyl-L-histidine has been studied by ^1H nuclear magnetic resonance spectroscopy and potentiometry. These peptides were chosen as models for the N-terminal region of the β chains of hemoglobin. Zinc(II) binding sites were identified by the effect of binding on chemical shifts of resonances for carbon-bonded protons, and formation constants of the complexes were determined from pH titration data. At physiological pH and above, complexes form which are in slow exchange with the free peptide on the NMR time scale. Correlation of pH titration results with the intensities of the slow-exchange resonances indicate that they correspond to complexes from which an extra equivalent of protons has been titrated. The chemical shifts of the resonances for the complexed ligand provide evidence that the extra proton is titrated from the amide group of the histidine residues, with Zn(II) binding to the N-terminal amino nitrogen, the deprotonated histidine amide nitrogen, and the imidazole 1-nitrogen. The results are discussed with reference to the binding of Zn(II) by hemoglobin.

The binding of zinc(II) by hemoglobin causes an increase in hemoglobin oxygen affinity.[1,2] This effect, the finding that sickel cell anemia frequently correlates with a decrease in the erythrocyte concentration of zinc,[3] and the proposed use of zinc for the treatment of sickel cell anemia[4] have stimulated interest in the molecular details of the binding of Zn(II) by hemoglobin.[5-14] Much of this research has been concerned with identifying the Zn(II) binding sites of hemoglobin, with the objective of elucidating the mechanism by which the oxygen affinity is increased.[15] From the effect of pH on the apparent Zn(II) binding constant, Rifkind and Heim concluded that a histidine residue is involved in the binding of Zn(II).[7] Direct spectroscopic evidence for the involvement of at least one histidine residue was obtained from ^1H nuclear magnetic resonance (NMR) studies of the interaction of Zn(II) with intact human erythrocytes;[12] however, it was not possible to identify the specific histidine residue(s) involved.

In view of the importance of histidine as a binding site for zinc in hemoglobin, and in numerous enzymes,[16-21] we are studying the binding of Zn(II) by selected histidine-containing peptides. We report here the results of ^1H NMR and potentiometric studies of the complexation of Zn(II) by two dipeptides (I and II) which have histidine in the second position of the peptide chain. These



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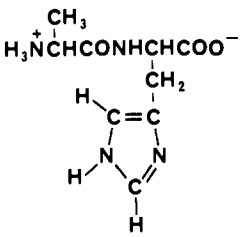

II

peptides were chosen as models for histidine-β2 and the N-terminal region of the β chain of hemoglobin. Zinc(II) binding sites were

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identified by ^1H NMR, and formation constants for the complexes were determined from pH titration data.

An important result of this research is that spectroscopic evidence has been obtained for the binding of Zn(II) by deprotonated peptide nitrogen. Metal-induced ionization of hydrogen from the peptide nitrogen with subsequent metal binding to the deprotonated nitrogen is a characteristic feature of the coordination chemistry of Pd(II), Cu(II), Ni(II), Co(II), and Co(III) with peptides,[22,23] but definitive evidence for similar reactions with Zn(II) has been lacking.[24]

Experimental Section

Chemicals. Glycyl-L-histidine and L-alanyl-L-histidine (Sigma Chemical Co.) and $Zn(NO_3)_2 \cdot 6H_2O$ (Baker Chemical Co.) were used as received. KNO_3 was twice-recrystallized from hot water before use. Titrant KOH solutions were prepared from a DILUT-IT kit (Baker Chemical Co.) and standardized by titration of potassium hydrogen phthalate. Solutions were prepared in doubly-distilled deionized water.

NMR Measurements. ^1H NMR spectra were obtained at 360 MHz and 25 °C with a Bruker WM-360 spectrometer operating in the pulse/Fourier transform mode. For measurements made on D_2O solutions, the solvent resonance was used for the lock signal. For measurements made on H_2O solutions, 1% D_2O was added to provide a lock signal. Typically 40–120 free induction decays were coadded. Chemical shifts were measured relative to internal *tert*-butyl alcohol but are reported relative to the methyl resonance of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). To avoid the dynamic range problem, the H_2O resonance was reduced in intensity with a selective saturation pulse prior to the nonselective observation pulse in measurements on H_2O solutions.

pH Measurements were made at 25 °C with Fisher Model 520 or Orion Model 701A pH meters equipped with a Fisher microcombination electrode or with a Philips GAT130 low-resistance-glass/Philips R44/2-SD/1 inverted-glass-sleeve, double-junction, saturated calomel reference electrode pair. The electrode pair was used in the measurement of pH titration data from which formation constants were evaluated. The outer liquid junction solution of the reference electrode was 0.30 M KNO_3. The pH meter and electrodes were calibrated by using buffer solutions of pH 4.008 and 6.865, prepared according to NBS specifications.[25] For the evaluation of formation constants from pH titration data, the pH meter and electrodes were calibrated in terms of hydrogen ion concentration by titration of strong acid solution, as described previously.[26] Meter readings from measurements on D_2O solutions were converted to pD values with the relationship pD = pH meter reading +0.40.[25] No attempt was made to correct for deuterium isotope effects in the 99% H_2O/1% D_2O solvent.

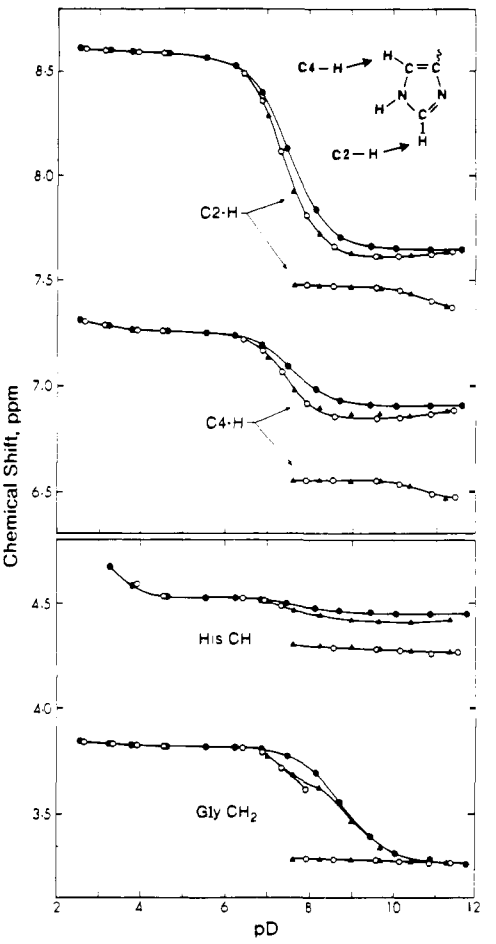

Figure 1. pD dependence of the chemical shifts of imidazole C2–H and C4–H protons, the histidyl α-CH proton, and the glycyl CH_2 protons of glycylhistidine in D_2O solutions containing 0.005 M Gly-His (-●—●-), 0.005 M Gly-His and 0.005 M $Zn(NO_3)_2$ (-○—○-), and 0.005 M Gly-His and 0.0025 M $Zn(NO_3)_2$ (-△—△-). 0.15 M $NaNO_3$, 25 °C.

Solution Preparation. A stock solution of Zn(II) was prepared by adding $Zn(NO_3)_2 \cdot 6H_2O$ to 99% H_2O/1% D_2O containing HNO_3 and KNO_3 to give a final Zn(II) concentration of 0.01 *m* and nitrate concentration of 0.300 *m*. The solution was standardized by gravimetric titration with EDTA in pH 10 ammonical buffer using Eriochrome Black T or pyrocatechol violet indicator. Stock solutions of the various peptides were prepared by adding solid ligand to 99% H_2O/1% D_2O containing KNO_3 and HNO_3. Peptide concentrations were determined by pH titration with data evaluation by the computer programs MINIQUAD81[27] or ACBA.[28]

For the ^1H NMR measurements, aliquots of the standardized Zn(II) and peptide solutions were placed in the titration cell,[26] and the initial pH of the resulting solution was adjusted to between 2 and 4 with 99% H_2O/1% D_2O containing HNO_3 and KNO_3 (total NO_3^- concentration of 0.30 M). *tert*-Butyl alcohol was added as an internal chemical shift reference at a concentration of $\sim 5 \times 10^{-4}$ M. The pH of the solution was then adjusted by titration with concentrated KOH, and ~0.5-mL samples were removed at selected pH values for NMR measurements. Typically 10–25 samples were removed over the pH range 2–11. The concentrations of ligand were in the range 2–20 mM with ligand-to-metal ratios of 1:1 to 2:1. Solutions were bubbled with argon for 5 min before starting the titration and were continually bathed in argon during the titration to minimize absorption of CO_2.

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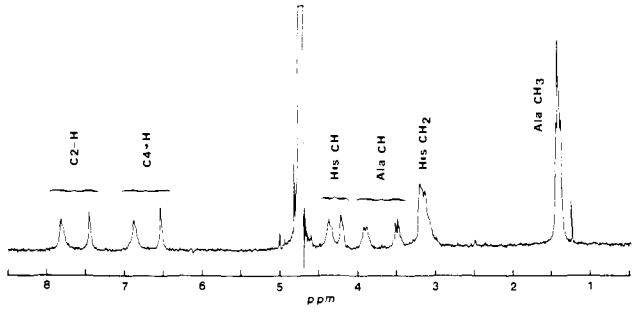

Figure 2. ^{1}H NMR spectrum of a solution containing 0.010 M Ala-His and 0.010 M $Zn(NO_3)_2$ in D_2O at pD = 7.4.

Solutions in D_2O were prepared directly from the requisite amounts of solid $NaNO_3$, ligand, and $Zn(NO_3)_2$. D_2O was added, and NMR samples were removed as the pD was adjusted with concentrated DNO_3 or NaOD solution.

pH titrations were done in an automated mode with the IBM personal computer-based equilibrium titrator described previously.[26] The acid concentration of the solvent (99% H_2O/1% D_2O containing HNO_3 and KNO_3 at a total NO_3^- concentration of 0.3 *m*) was determined by titration with standardized KOH. Typically the HNO_3 concentration was ~10 m*m*. Calibration parameters for the pH meter in terms of hydrogen ion concentration were calculated from the titration data[26] with the programs ACBA[28] or KINET.[29]

The experimental procedure used to obtain pH titration data for the determination of peptide acid dissociation constants and zinc(II)–peptide formation constants involved first titrating an aliquot of the stock peptide solution in solvent with 1.002 M KOH to a predetermined pH (e.g., pH 10) and then acidic Zn(II) stock solution was added to the basic peptide solution in the titration cell and the titration repeated. Peptide acid dissociation constants were determined from the peptide pH titration data with the programs ACBA[28] or MINIQUAD81,[26,27] while zinc(II)–peptide formation constants were calculated with MINIQUAD81.[26,27] Only those zinc(II)–peptide complexes which accounted for at least 5% of the total peptide at some pH in the pH range 4–9.5 were kept in the model as it was refined.

Results

NMR Results. ^{1}H NMR spectra were measured as a function of pD for each of the peptides alone in solution and in solutions containing $Zn(NO_3)_2$. Chemical shift data for free glycyl-L-histidine (Gly-His) and for Gly-His in solutions containing Zn(II) at Gly-His/Zn^{II} ratios of 1:1 and 2:1 are shown in Figure 1. In the absence of Zn(II), the chemical shift of the resonance for the proton on the α carbon of the histidine residue (His CH) changes over the pD range 3–5, reflecting titration of the adjacent $-CO_2H$ group. The pD dependence of this chemical shift is identical in the presence of Zn(II), indicating no detectable complexation of Zn(II) by the deprotonated carboxylate group. Over the pD range 6–9, the chemical shifts of the imidazole C2–H and C4–H resonances change, reflecting titration of the imidazolium group, while the chemical shift of the Gly CH_2 resonance shifts over the pD range 7–11 due to titration of the glycyl ammonium group.[30] The His CH resonance shifts over these same pD ranges, and the shift presumably reflects a combination of effects from titration of the imidazolium and ammonium groups.

In the presence of Zn(II), resonances whose chemical shifts are pD-dependent are observed for these carbon-bonded protons and, in addition, a second set of resonances is observed at pD greater than ~7.5. The chemical shifts of the resonances in the second set are pD-independent up to pD ~9.5 and then they shift with increasing pD. Chemical shift data are given for the Zn^{II}–Gly-His system in Figure 1. Similar spectra were observed for the Zn^{II}–Ala-His system, as illustrated by the spectrum in Figure 2 which is for a solution containing 0.010 M $Zn(NO_3)_2$ and 0.010 M Ala-His in D_2O at pD 7.4. The intensity of the resonances in the second set increases as the pD is increased at the expense of the resonances in the other set, as shown by the spectra for the Zn^{II}–Gly-His system in Figure 3.

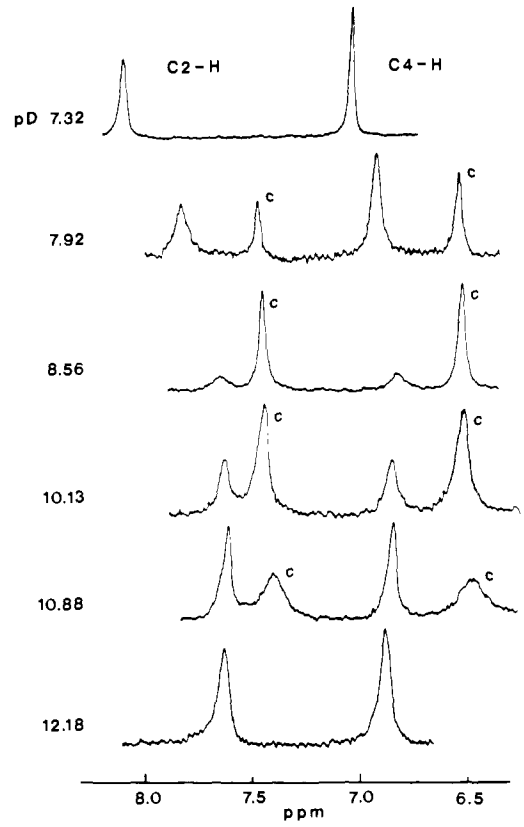

Figure 3. Imidazole region of the ^{1}H NMR spectra of a D_2O solution containing 0.005 M Gly-His, 0.005 M $Zn(NO_3)_2$, and 0.15 M $NaNO_3$ as a function of pD. c indicates resonances of Gly-His in the complex $ZnLH_{-1}$. See text for discussion.

The results in Figures 1–3 indicate the formation of several Zn^{II}–Gly-His and Zn^{II}–Ala-His complexes. The pD dependence for the chemical shifts of the C2–H and C4–H resonances indicates binding to the imidazole group starting at pD ~6.5, while the pD dependence for the Gly CH_2 resonance indicates binding to the ammonium group starting at pD ~7. The observations of resonances whose chemical shifts are pD-dependent for these protons indicates fast exchange of peptide between the free and complexed forms. However, the second set of resonances which is present over the pD range 7.5–12 indicates formation of another complex which is kinetically stable on the NMR time scale. In the following, those resonances from the peptide which is in fast exchange between free and complexed forms will be identified as fast-exchange resonances while those from the peptide in the kinetically stable complex will be referred to as complex resonances. The change in chemical shift of the complex resonances for C2–H and C4–H and the simultaneous decrease in their intensity at pD >9.5 was observed for both the Zn^{II}–Gly-His (Figures 1 and 3) and Zn^{II}–Ala-His systems. These changes suggest formation of a mixed zinc(II)–peptide–hydroxy complex at pD > 9.5 followed by dissociation of the complex with the formation of $Zn(OH)_4^{2-}$.

The spectra in Figure 3 are for a 1:1 Gly-His/Zn^{II} ratio. At a 2:1 ratio, the integrated intensity of the complex resonances is never more than ~50% of the total, indicating little if any formation of kinetically stable complexes in which the ligand-to-Zn(II) ratio is 2:1. Both the fast-exchange resonances and the complex resonances for C2–H and C4–H in Figure 3 are relatively broad. The series of spectra in Figure 4 for a pD 7.92 solution containing 0.005 M $Zn(NO_3)_2$ and Gly-His indicate that the broadening is due to exchange of Gly-His between the fast-exchange and complex forms.

Potentiometric Results. Solutions containing peptide and peptide + Zn(II) were titrated to determine peptide acid dissociation constants and formation constants for the zinc(II)–peptide complexes. Representative titration data are shown in Figure 5. The acid dissociation constants obtained for Gly-His and Ala-His

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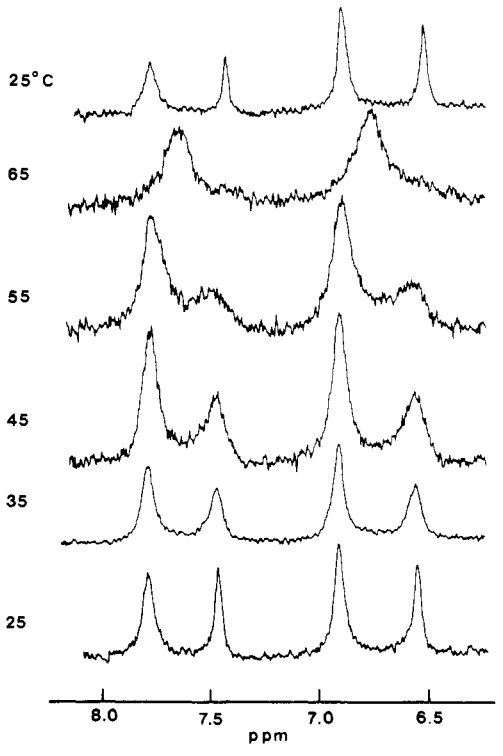

Figure 4. Imidazole region of ^1H NMR spectra of a pD = 7.92 solution containing 0.005 M Gly-His, 0.005 M $Zn(NO_3)_2$, and 0.15 M $NaNO_3$ as a function of temperature. The top spectrum (25 °C) was measured after the 65 °C spectrum was measured.

Table I. Acid Dissociation Constants for Gly-His and Ala-His[a,b]

	pK_{A1}	pK_{A2}	pK_{A3}
Gly-His	2.61	6.77	8.14
Ala-His	2.73	6.76	8.08

[a] In 0.300 *m* KNO_3. 25 °C. [b] Standard deviations are 0.01 p*K* unit or less.

are given in Table I. Preliminary analysis of titration data for the zinc(II)–peptide solutions indicated titration of more protons than for peptide alone, as has been observed previously.[24a-c] On average, 1.04 mmol of additional protons was titrated per millimole of Zn(II) in solutions having Gly-His/Zn^{II} and Ala-His/Zn^{II} ratios of 1:1 or larger.

Formation constants were evaluated from titration data starting at pH 4 up to a maximum of pH 9.5. The formation constants are defined by eq 1 and 2 where L is fully deprotonated ligand,

$$pZn + qL + rH \rightleftarrows Zn_pL_qH_r \quad (1)$$

$$\beta_{pqr} = \frac{[Zn_pL_qH_r]}{[Zn]^p[L]^q[H]^r} \quad (2)$$

i.e., ligand from which carboxylic acid, imidazolium and ammonium protons have been titrated. Zinc also forms hydroxy complexes,[31] and it was found that the fits were improved when the species $Zn(OH)^+$ and $Zn(OH)_2$ were included in the formation constant calculations. Formation constants (eq 3 and 4) for $Zn(OH)^+$ and $Zn(OH)_2$ were determined to be $\log \beta_1 = 6.62$ and $\log \beta_2 = 11.44$ from separate titrations of 0.009 M $Zn(NO_3)_2$ in 0.3 M KNO_3.[32] Binuclear species of the type $Zn_2(peptide)_x$ were

$$\beta_1 = \frac{[Zn(OH)^+]}{[Zn^{2+}][OH^-]} \quad (3)$$

$$\beta_2 = \frac{[Zn(OH)_2]}{[Zn^{2+}][OH^-]^2} \quad (4)$$

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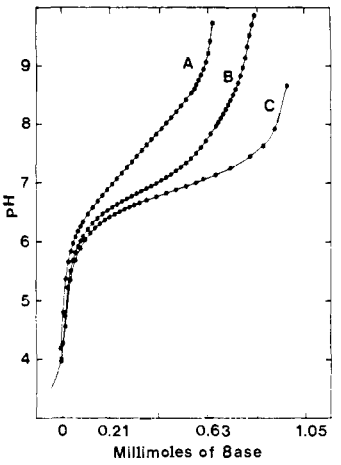


Figure 5. Representative potentiometric titration curves. (A) 0.308 mmol Gly-His, (B) 0.308 mmol Gly-His and 0.123 mmol $Zn(NO_3)_2$, and (C) 0.309 mmol Gly-His and 0.284 mmol $Zn(NO_3)_2$. Half the experimental points are shown. The solid curves through the experiment points are theoretical curves calculated with the pK_A values for Gly-His in Table I and the formation constants for the Zn^{II}–Gly-His complexes in Table II.

repeatedly rejected by MINIQUAD81 and were eliminated from the model. The formation constants calculated from the titration data are given in Table II.

Complexes with $r = -1$ are those from which an additional proton has been titrated. The fractional concentrations of complexed Gly-His obtained from the intensities of the slow-exchange complex resonances for the imidazole C2–H and C4–H protons in the ^1H NMR spectra of Zn^{II}–Gly-His in 1% D_2O/99% H_2O solutions at the concentrations used in the formation constant determinations were the same, within experimental error,[33] as the fractional concentrations predicted for the $ZnLH_{-1}$ complex in species distributions calculated with the formation constants in Table II. This indicates that the slow-exchange complex resonances are for the complex $ZnLH_{-1}$.

Discussion

The pH titration experiments are in agreement with previous studies[24a-c] of the complexation of Zn(II) by Gly-His in that an additional equivalent of proton is titrated per Zn^{II}–Gly-His complex. A more quantitative comparison is possible only with the results of Farkas et al.[24c] who found that their titration data could be fitted by a model similar to that deduced here. Considering that different conditions were used in their experiments and that some of the complexes in the model are present in low abundance, the agreement between our results and theirs (footnote c, Table II) is reasonable. Although the details of the models used in the previous studies are different, in each case the titration of an additional equivalent of proton is interpreted to indicate formation of an amide-deprotonated Zn^{II}–Gly-His complex.[34] In the present study, this complex corresponds to the species of composition $ZnLH_{-1}$. The agreement between the fractional concentrations predicted for this species using the formation

(32) The titration of Zn^{2+} with OH^- was stopped before precipitation of $Zn(OH)_2$ occurred. Incipient precipitation could be detected by drifting pH meter readings before it could be detected visually.

(33) The average of the absolute difference between predicted and observed fractional concentrations was 5.9%. The estimated uncertainty of the fractional concentrations determined by NMR is 10%.

(34) Other possibilities for the group from which the additional equivalent of proton is titrated include a coordinated water molecule and the pyrrole group of the Zn(II)-coordinated imidazole side chain. The first can be ruled out by comparison with the pK_A values for the titration of a water proton from the Zn(II) complexes of nitrilotriacetic acid and β,β',β''-triaminotriethylamine (10.0 and 11.1, respectively) (Rabenstein, D. L.; Blakney, G. *Inorg. Chem.* **1973**, *12*, 128–132). Also, the chemical shift data for the complexed resonances at pH >9.5 (Figure 1) suggest titration of a water molecule coordinated by $ZnLH_{-1}$ at pH > 9.5. Titration of the pyrrole group of the Zn(II)-coordinated imidazole side chain is unlikely since the pK_A for Zn(II)-induced pyrrole ionization is estimated to be about 13 (Martin, R. B. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 4346–4347).

Table II. Formation Constats of Zinc(II)–Peptide Complexes[a,b]

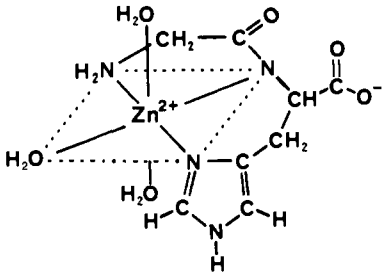
	$\log \beta_{11-1}$	$\log \beta_{110}$	$\log \beta_{111}$	$\log \beta_{12-1}$	$\log \beta_{120}$
Zn^{II}–Gly-His[c]	−2.14 ± 0.04	3.85 ± 0.02	11.54 ± 0.02	1.31 ± 0.01	8.85 ± 0.01
Zn^{II}–Ala-His	−3.6 ± 0.1	3.5 ± 0.5	10.3 ± 0.1	0.4 ± 0.1	7.8 ± 0.1

[a] In 0.300 *m* KNO_3. 25 °C. [b] Values given are the weighted averages and weighted standard deviations of values determined from six independent experiments. [c] The corresponding values reported by Farkas et al.[24c] are −2.75, 3.98, 10.87, 0.37, and 8.03, respectively, and, in addition, $\log \beta_{11-2} = -12.66$.

constants determined in the present work for the Zn^{II}–Gly-His system and the fraction of the total imidazole resonance intensity in the imidazole resonances at 7.46 and 6.55 ppm indicates that these resonances are due to the species $ZnLH_{-1}$.[33]

The chemical shifts of resonances for the carbon-bonded protons of the $ZnLH_{-1}$ complexes of Gly-His and Ala-His provide information about the nature of the complex. The chemical shifts of the resonances for the C2–H and C4–H protons of the imidazole group (Figure 1) indicate binding to the imidazole ring, while the chemical shifts of the glycyl CH_2 protons of the complex indicate binding to the amino nitrogen. Of particular interest are the chemical shifts of the His CH resonances. In the Zn–Gly-His complex, this resonance is 0.156 ppm to lower frequency from the resonance for deprotonated Gly-His, while in the Zn–Ala-His complex it is 0.212 ppm to lower frequency. These changes in chemical shift are evidence for binding of Zn(II) to deprotonated amide nitrogen in the $ZnLH_{-1}$ complexes. Estimates of the shifts to be expected if they were due to binding to the amino nitrogen, the amide carbonyl oxygen, or the carboxylate oxygen can be obtained from results for zinc(II)–glycine peptide complexes. The resonance for the CH_2 protons of the middle glycyl residue of the zinc(II)–glycylglycylglycine complex, in which Zn(II) is chelated by the amino nitrogen and the carbonyl oxygen of the N-terminal glycyl residue, is shifted 0.092 ppm to higher frequency from the resonance for deprotonated glycylglycylglycine, while the resonance for the CH_2 protons of the C-terminal residue of the carboxylate-coordinated Zn(II) complex of glycylglycine is shifted 0.090 ppm to higher frequency from the resonance for deprotonated glycylglycine.[35] The shift of the His–CH resonances for the $ZnLH_{-1}$ complexes in the opposite direction suggests that the shift is not due to complexation at the amino nitrogen, the amide carbonyl oxygen, or the carboxylate oxygen. Coordination at the imidazole group as the source of the shift in the His–CH resonance can be ruled out by considering the effect of binding on the resonances for the protons on the β carbon of histidine. The shifts, 0.113 and 0.132 ppm to high frequency from the resonances for deprotonated Gly-His and Ala-His, respectively, are opposite in direction and smaller in magnitude than those for the resonances of the His CH protons which are one bond further from the imidazole group.

The chemical shift data for the glycyl, histidyl, and imidazole protons taken together thus indicate that the structure of the $ZnLH_{-1}$ complex for the Zn–Gly-His system is



The NMR data for the Zn–Ala-His system are consistent with the analogous complex for the Zn–Ala-His system.

The chemical shift data in Figure 1 indicate that there is binding to the amino and/or imidazole groups in the more labile complexes which also form. The formation constants for the ZnL and ZnHL ($Zn^{2+} + HL \rightleftarrows ZnHL$; $K_f = K_{A3}\beta_{111}$) complexes are similar in magnitude to those for the Zn(II) complex of imidazole[36] (log $K_f = 2.57$) and the amino-coordinated Zn(II) complex of glycylglycine[35] (log $K_f = 3.13$), which suggests simple monodentate binding in these complexes.

Although the effective dielectric constant in hemoglobin will be different than in aqueous solution, it is of interest to compare the formation constants measured for the zinc(II)–peptide complexes with apparent association constants reported for the binding of Zn(II) by hemoglobin. The apparent Zn(II) association constants depend on the state of the hemoglobin and the pH; the apparent association constant for Zn(II) with oxyhemoglobin is 1.3×10^7 M^{-1} at pH 7.2 and 2 °C[7] and 1.9×10^6 M^{-1} at pH 6.83–7.11 and 20 °C,[9] while that with carbomonoxyhemoglobin is 1.1×10^6 M^{-1} at pH 7.0 and 5×10^5 M^{-1} at pH 6.6, both at 20 °C.[9] For deoxyhemoglobin, the apparent association constant is 1.8×10^5 M^{-1} at pH 7.42 and 20 °C.[9] For comparison, apparent association constants for the $ZnLH_{-1}$ complexes, which are the most abundant complexes at pH 7.4, can be calculated from the formation constants in Table II. The apparent association constant is for the reaction

$$Zn^{II} + L_f \rightleftarrows ZnLH_{-1} \qquad (5)$$

where L_f includes all the various protonated forms of the free ligand. $K_{app} = \beta_{11-1}\alpha_2/[H^+]$ where α_2 is the fraction of free ligand in the fully deprotonated form.[37] The apparent association constants for the $ZnLH_{-1}$ complexes at pH 7.4 are 2.3×10^4 M^{-1} for the Gly-His complex and 1.0×10^3 M^{-1} for the Ala-His complex. These values are considerably smaller than the apparent association constants for hemoglobin, suggesting either that His β-2 and the N-terminal region of the β chains is not the Zn(II) binding site in human hemoglobin or, if it is, the Zn(II) must be simultaneously coordinated to additional donor groups to account for the extra stability. This indication that His β-2 is not the Zn(II) binding site in human hemoglobin is consistent with the finding of Rifkind and Heim[7] that the binding of Zn(II) is the same for human and bovine hemoglobin, even though the second residue in the β chain of bovine hemoglobin is leucine.[38]

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Registry No. Glycyl-L-histidine, 2489-13-6; L-alanyl-L-histidine, 3253-17-6; zinc, 7440-66-6.

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