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Nuclear Magnetic Resonance Studies of the Solution Chemistry of Metal Complexes. 22. Complexation of Zinc by the Growth-Modulating Tripeptide Glycylhistidyllysine

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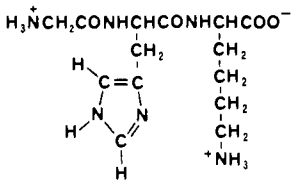
The binding of zinc by the tripeptide glycyl-L-histidyl-L-lysine (GHL) has been studied by ^1H NMR spectroscopy and by potentiometric titration. At physiological pH, resonances are observed for two kinetically stable complexes, and binding sites in these complexes have been identified from the chemical shifts of carbon-bonded protons of complexed GHL. Formation constants have been determined for Zn(II)–GHL complexes from pH titration data. Proton balance considerations indicate that an extra proton is titrated from the Zn(II)–GHL complexes, while the NMR results indicate that the extra proton is titrated from an amide group. It is concluded from chemical shift data that the GHL in one of the two kinetically stable complexes is tridentate, with Zn(II) coordinated by the deprotonated glycyl amino nitrogen, the deprotonated amide nitrogen of the glycyl–histidyl peptide bond, and the imidazole 1-nitrogen, while the amino group of the lysine side chain is protonated. The results are discussed with reference to the synergistic alteration of growth patterns of hepatoma cells by GHL and Zn(II).[5]

Introduction

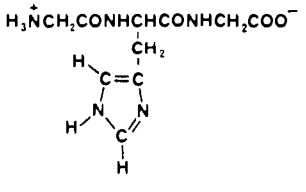
Glycyl-L-histidyl-L-lysine (GHL) a tripeptide present in human plasma,[1] stimulates the growth or enhances the viability of a variety of cultured cells or organisms at nanomolar concentrations.[2,3]



I

GHL coisolates from serum with copper, and it is thought that it is the copper complex which exerts the biological activity of GHL.[4,5] It has been proposed that the glycyl and histidyl residues function as Cu(II) chelators while the lysine residue, which is essential for biological activity,[4,6] is involved in the recognition of a cell surface receptor.[3] In solution, the Cu(II) complex is monomeric and tridentate, with the glycyl amino nitrogen, the amide nitrogen of the glycyl–histidyl peptide bond, and the imidazole 1-nitrogen coordinated to Cu(II) while the lysine residue is free.[7]

GHL also acts synergistically with other metals, including zinc, to alter patterns of cell growth in monolayer cultures of a tumorigenic hepatoma cell line.[5] Since the results suggest it is the metal complexed form of GHL that exerts this biological activity, we have studied the complexation of Zn(II) by GHL in aqueous solution. Zn(II) binding sites have been identified by ^1H nuclear magnetic resonance (NMR) spectroscopy, and formation constants have been determined by pH titration. The results indicate that several Zn(II)–GHL complexes form at physiological pH, including one that is identical with the Cu(II)–GHL complex. For comparison, the complexation of Zn(II) by glycyl-L-histidyl-glycine (GHG) has also been studied by ^1H NMR.



II

Experimental Section

Chemicals. Glycyl-L-histidyl-L-lysine acetate, glycyl-L-histidylglycine (Sigma Chemical Co.) and $Zn(NO_3)_2 \cdot 6H_2O$ (Baker Chemical Co.) were used as received. KNO_3 was recrystallized twice 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.

Solution Preparation. A stock solution of GHL was prepared by adding solid GHL to 99% H_2O/1% D_2O containing KNO_3 and HNO_3. The GHL concentration was determined by pH titration, with data evaluation by the computer programs MINIQUAD81[8] or ACBA.[9] 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 titration with EDTA.

Solutions used in the ^1H NMR measurements on GHL and Zn(II)–GHL in 99% H_2O/1% D_2O were prepared by placing aliquots of the Zn(II) and GHL stock solutions in a titration cell and adjusting the pH to between 2 and 4 with 99% H_2O/1% D_2O containing HNO_3 and KNO_3 (0.30 *m* total NO_3^- concentration). *tert*-Butyl alcohol was added for an internal chemical shift reference at a concentration of $\sim 5 \times 10^{-4}$ M. The pH of the solution was then adjusted with concentrated KOH, and ~0.5-mL samples were removed at selected pH values over the pH range 2–11. The concentrations of GHL were in the 2–20 m*m* range at GHL-to-Zn(II) ratios of 1:1 to 2:1. Solutions were bubbled with argon before titration and bathed with argon during titration to minimize absorption of CO_2.

Solutions in D_2O were prepared directly from solid $NaNO_3$, glycyl-L-histidylglycine acetate, and $Zn(NO_3)_2$. D_2O was added, and samples for NMR measurement were removed as the pD was adjusted with concentrated DNO_3 or NaOD solution.

pH measurements were made at 25 °C with a Fisher Model 520 or Orion Model 701A pH meter equipped with a Fisher microcombination electrode or with a Philips GAT130 low-resistance glass electrode/Philips R44/2-SD/1 inverted glass-sleeve, double-junction, saturated calomel reference electrode pair. The outer liquid-junction solution of the reference electrode was 0.30 *m* KNO_3. The electrode pair was used in the measurement of pH titration data from which formation constants were evaluated. The pH meter and the electrode pair were calibrated in terms of hydrogen ion concentration by titration of a strong acid solution, as described previously.[10] Meter readings from measurements on D_2O

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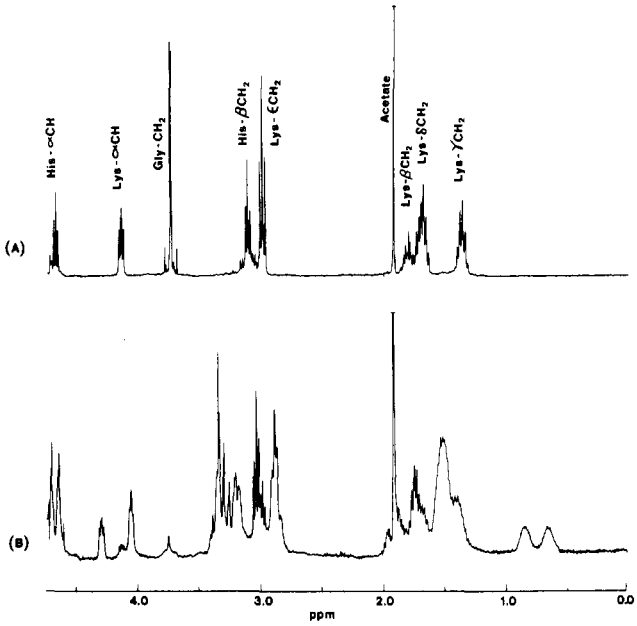

Figure 1. Portions of the ^1H NMR spectra of (A) 0.010 M GHL in D_2O at pD 7.5 and (B) 0.010 M GHL and 0.0095 M $Zn(NO_3)_2$ *in* D_2O at pD 7.5.

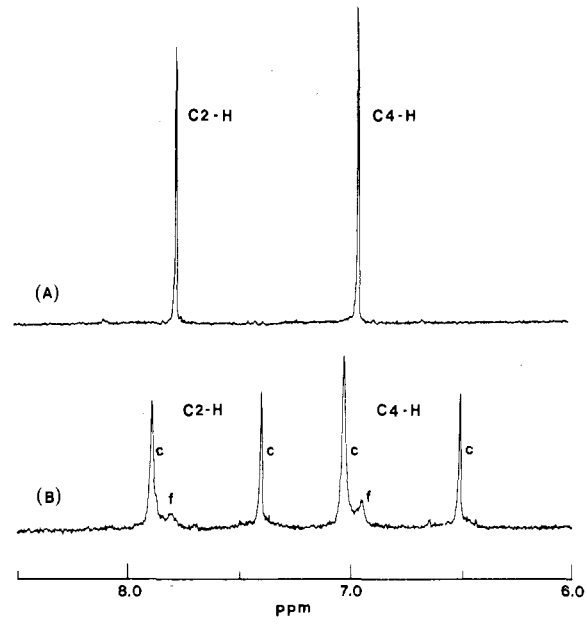

Figure 2. Imidazole region of the ^1H NMR spectra of (A) 0.010 M GHL in D_2O at pD 7.5 and (B) 0.010 M GHL and 0.0095 M $Zn(NO_3)_2$ in D_2O at pD 7.5. f indicates imidazole resonances of GHL in fast exchange between the free form and labile complexes and c indicates resonances of GHL in kinetically stable complexes.

solutions are converted to pD values with the relationship pD = pH meter reading +0.40.[11] No attempt was made to correct for deuterium isotope effects in the 99% H_2O/1% D_2O solvent.

pH Titrations. The automated equilibrium titrator used to obtain pH titration data has been described previously.[10] Titrations were done on GHL and GHL + $Zn(NO_3)_2$ in a solvent consisting of 99% H_2O/1% D_2O containing HNO_3 and KNO_3 at a total NO_3^- concentration of 0.30 *m*. The acid concentration of the solvent was determined by titration with standardized KOH and was typically ~45 m*m*.

pH titration data for the determination of GHL acid dissociation constants were obtained by titrating an aliquot of the stock peptide solution in solvent with KOH to a pH of ~10. pH titration data for the determination of Zn(II)–GHL formation constants were obtained by then adding acidic Zn(II) stock solution to the titrated GHL solution in the titration cell and repeating the titration. This procedure was used to minimize errors due to variations in the liquid-junction potential. Calibration parameters for the pH meter in terms of hydrogen ion concentration and GHL acid dissociation constants were obtained from the first titration with the programs ACBA[9] or MINIQUAD81[8] while the Zn(II)–GHL formation constants were calculated from the second titration with MINIQUAD81.[8] Only those Zn(II)–GHL complexes accounting for at least 5% of the total GHL at some pH in the pH range 4–9.5 were retained as the model was refined.

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 or at 400 MHz and 25 °C with a Bruker WH-400 spectrometer operating in either the rapid-scan cross-correlation mode[12,13] or 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, the solvent contained 1% D_2O for a lock signal.

Most measurements were made by the pulse/Fourier transform method. Typically, 100 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-sulfonic acid (DSS). The H_2O resonance from solutions in 99% H_2O/1% D_2O was reduced in intensity with a selective saturation pulse prior to the nonselective observation pulse.

The rapid-scan cross-correlation method was used to measure spectra of the 6.5–9.5 ppm region for some H_2O solutions so as to obtain resonances for amide protons, which are exchanging with the solvent protons. A scan time of 5 s was used, and typically 100 scans were coadded. Spectra were measured on nonspinning samples to avoid artifacts from sample spinning. Chemical shifts were measured relative to $CHCl_3$ in an external capillary (7.244 ppm vs. DSS). Susceptibility changes as the solution pH was varied were found to be small, and no corrections were made to measured chemical shifts.

Results and Discussion

The 0–4.75 ppm region of ^1H NMR spectra of (A) 0.010 M GHL in D_2O at pD 7.5 and of (B) 0.010 M GHL and 0.0095 M $Zn(NO_3)_2$ at pD 7.5 are shown in Figure 1. The 6.0–8.5 ppm regions of ^1H NMR spectra of the same solutions are shown in Figure 2. Comparison of the spectra for the two solutions reveals that two complexes, which are kinetically stable on the NMR time scale, are formed. This is indicated most clearly in spectrum B of Figure 2, where two resonances are observed for both the imidazole C2-H and C4-H protons of complexed GHL in addition to weaker resonances for the excess GHL. The chemical shifts of the two C2-H resonances for complexed GHL are 7.897 and 7.391 ppm, while those of the C4-H resonances are 7.032 and 6.512 ppm. The two resonances at 7.897 and 7.032 ppm are of similar intensity, as are those at 7.391 and 6.512 ppm. The resonances of the 7.897, 7.032 ppm pair are of slightly greater intensity than those of the other pair, leading to the assignment of the 7.897, 7.032 ppm pair to one GHL complex (complex **1**) and the 7.391, 6.512 ppm pair to a second complex (complex **2**).

The resonances for the other carbon-bonded protons of GHL (spectrum B in Figure 1) also indicate the formation of two complexes. Chemical shifts of selected resonances for both complexes, and for free GHL at various stages of protonation and at pD 7.4, are summarized in Table I.

Resonances in spectra measured as a function of pD for D_2O solutions of 0.003 M GHL and 0.003 M Zn(II)–GHL indicate that the complexation of GHL by Zn(II) is pD-dependent. As the pD of the GHL solution is increased, the resonances for the C2-H and C4-H protons start to shift at pD ~5, due to titration of the imidazolium group. For the Zn(II)–GHL solution, the resonances start to shift at lower pD (~4.5), indicating displacement of the imidazolium proton with binding to Zn(II) to form a kinetically labile complex. When the pD is increased to ~6.5, resonances for the kinetically stable complexes **1** and **2** are detectable. As the pD is increased further, these resonances increase in intensity while the intensity of the exchange-averaged resonances for the free ligand and kinetically labile complexes decreases. The chemical shifts of the resonances for the C2-H and C4-H protons of complexes **1** and **2** are constant over the pD range 6.5–9.5. Also, the chemical shift of the resonance for acetate

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Table I. Chemical Shifts of Carbon-Bonded Protons of Glycyl-L-histidyl-L-lysine and of Zinc Complexes of Glycyl-L-histidyl-L-lysine[a,b]

	C2-H	C4-H	Gly CH_2	His α-CH	His β-CH_2	Lys α-CH	Lys ϵ-CH_2
H_4L^{3+}[c]	8.637	7.334	3.855	*d*	3.240	4.371	2.991
H_3L^{2+}[e]	8.609	7.349	3.855	*d*	3.270	4.146	2.994
$HL^{\pm}$[f]	7.672	6.946	3.333	*d*	3.067	4.129	2.960
L^-[g]	7.669	6.943	3.308	*d*	3.067	4.117	2.554
pD 7.4	7.801	7.011	3.731	4.667	3.108	4.129	2.979
complex **1**	7.897	7.032	3.338	4.635	3.185	4.047	2.882
complex **2**	7.391	6.512	3.324	4.693	*h*	4.289	3.031

[a] Chemical shifts are for the center of multiplet patterns. [b] In ppm vs. DSS. [c] pD 1.04. Fully protonated GHL. [d] Not observed; overlapped by HDO resonance. [e] pD 4.70. Glycyl and lysine amino groups and imidazole group protonated. [f] pD 9.57. Lysine amino group protonated. [g] pD 12.90. [h] Not resolvable but in the 3.18 ppm region.

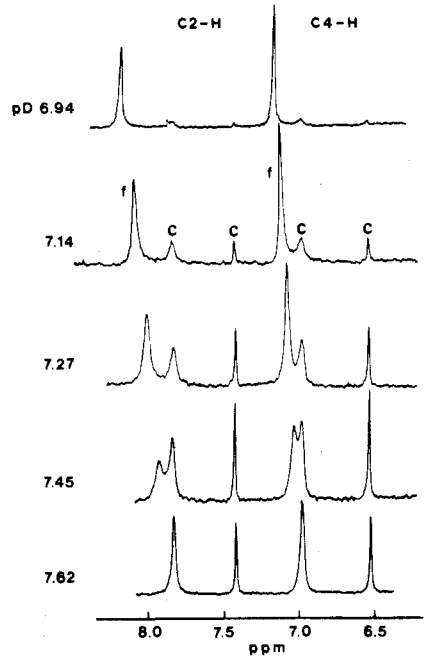

Figure 3. Imidazole region of ^1H NMR spectra of D_2O solutions containing 0.005 M GHG, 0.005 M $Zn(NO_3)_2$, and 0.15 M $NaNO_3$ as a function of pD. f indicates imidazole resonances of GHG in fast exchange between the free form and labile complexes and c indicates resonances of GHG in kinetically stable complexes.

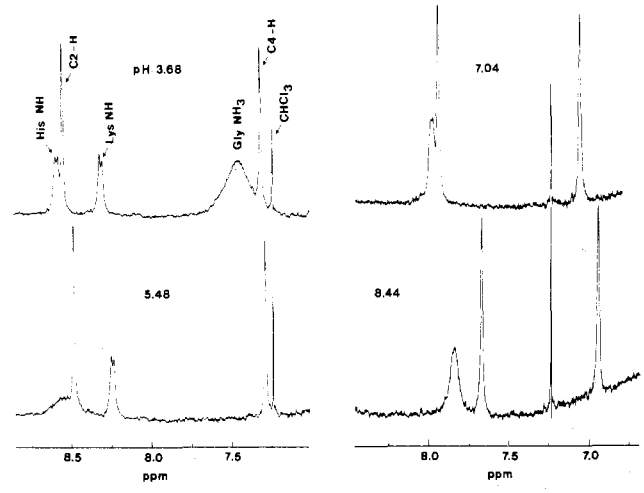

Figure 4. Portions of the ^1H NMR spectra of 0.010 M GHL in 1% D_2O/99% H_2O (0.15 M $NaNO_3$) at several pH values. Spectra were measured by the rapid-scan cross-correlation method.

(1.908 ppm) is the same in both solutions, indicating no detectable binding of Zn(II) by acetate in the presence of GHL.

Identical behavior was observed for the imidazole C2-H and C4-H protons of the Zn(II)–glycylhistidylglycine system, as shown by the spectra in Figure 3. These spectra are for the C2-H and C4-H protons of GHG in a D_2O solution containing 0.005 M GHG and $Zn(NO_3)_2$ and 0.15 M $NaNO_3$. The resonances for GHG in the kinetically stable complexes are just detectable at pD 6.94, and increase in intensity as the pD is increased until they are the only resonances detectable at pD 7.62. The chemical shifts of the resonances for the C2-H protons of the kinetically stable complexes of GHG are 7.846 and 7.434 ppm while those of the resonances for the C4-H protons are 6.986 and 6.528 ppm. From line widths and relative intensities, the 7.846, 6.986 ppm pair of resonances is assigned to one complex and the pair at 7.434, 6.528 ppm to a second complex. The chemical shifts of the imidazole protons of these two complexes are essentially identical with those observed for the Zn(II)–GHL system, suggesting that the same two kinetically stable Zn(II) complexes form with GHL and GHG. The chemical shifts of the imidazole resonances for complex **2** of both GHL and GHG are also very similar to those for kinetically stable Zn(II) complexes of glycyl-L-histidine (7.46 and 6.55 ppm) and L-alanyl-L-histidine (7.49 and 6.55 ppm),[14] suggesting further that complex **2** of GHL and GHG is similar to the Zn(II) complexes of glycyl-L-histidine and L-alanyl-L-histidine. The glycyl-L-histidine and L-alanyl-L-histidine complexes are tridentate, with Zn(II) binding to the N-terminal amino nitrogen, the deprotonated amide nitrogen, and the imidazole 1-nitrogen.[14-17]

Chemical shifts of the resonances for the Lys ϵ-CH_2 protons of complexes **1** and **2** are similar to those given in Table I for GHL in the H_4L^{3+}, H_3L_{2+}, and HL forms, indicating that in complexes **1** and **2** the amino group of the lysine side chain is protonated and not Zn(II) coordinated. Estimates of the magnitude of the shift to be expected upon replacement of an ammonium proton by Zn(II) can be obtained from the results in Table I for the Gly CH_2 resonance (~0.53 ppm for complex **2**) and from the shifts of the resonance for the CH_2 protons of ethylenediamine upon complexation by the Zn(II)–nitrilotriacetic acid (0.56 ppm) complex.[18]

To elucidate further the nature of the complexes of Zn(II) with GHL, the 6–9.5 ppm regions of the ^1H NMR spectra of GHL and of Zn(II)–GHL in H_2O solution were measured by the rapid-scan cross-correlation method so as to observe resonances for nitrogen-bonded protons.

The spectra in Figure 4 are for 0.010 M GHL in H_2O solution containing 0.15 M $NaNO_3$ while those in Figure 5 are for 0.020 M GHL and 0.020 M $Zn(NO_3)_2$. At pH 3.68 (Figure 4), resonances are observed for the imidazole C2-H and C4-H protons, for the glycyl (and/or lysine) ammonium protons, and for the NH protons of the two peptide groups. The resonances for the two peptide NH protons are assigned by analogy with the assignments for the peptide NH protons of GHG, where a doublet is observed for the His NH and a triplet for the NH of the C-terminal glycine. The Gly NH_3 resonance in Figure 4 is broadened by exchange of protons between the ammonium group and solvent. As the pH is increased, the rate of proton exchange increases and this resonance disappears, as does the resonance for the His NH. In

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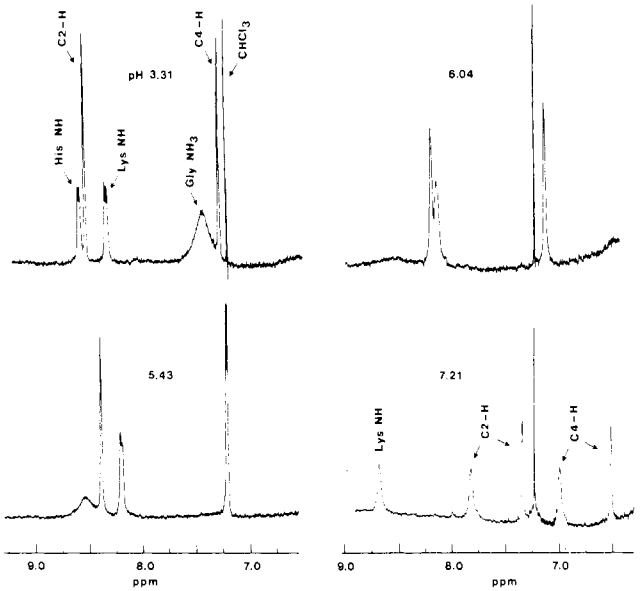


Figure 5. Portions of the ^1H NMR spectra of 1% D_2O/99% H_2O solutions containing 0.020 M GHL, 0.020 M $Zn(NO_3)_2$, and 0.15 M $NaNO_3$ at several pH values. Spectra were measured by the rapid-scan cross-correlation method.

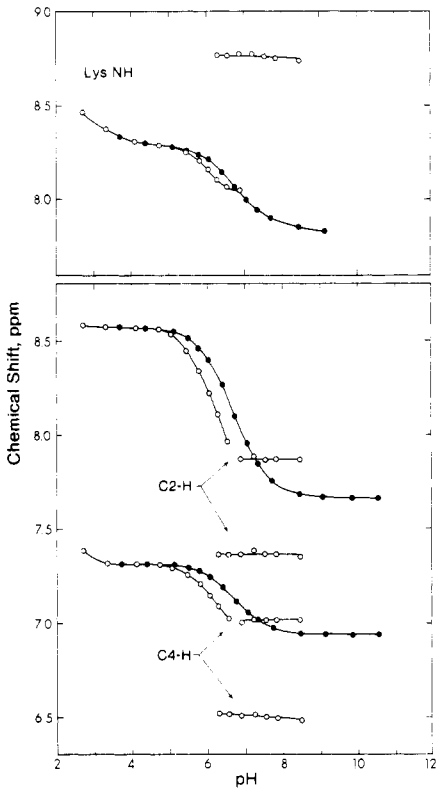


Figure 6. pH dependence of the chemical shifts of the imidazole C2-H and C4-H protons and lysine amide (Lys NH) proton of GHL in 1% D_2O/99% H_2O solutions containing 0.010 M GHG (●) and 0.020 M GHL and 0.020 M $Zn(NO_3)_2$ (○) in 0.15 M $NaNO_3$ at 25 °C.

contrast, the Lys NH resonance is observed even at pH 8.44. The pH dependence of the chemical shifts of the C2-H, C4-H, and Lys NH resonances is shown in Figure 6.

In the presence of an equimolar concentration of Zn(II), the behavior of the resonances for these protons is different (Figures 5 and 6). At pH 5.43, the resonances for C2-H, C4-H, and Lys NH are all shifted from those observed in the absence of Zn(II) due to exchange of GHL between free and kinetically labile complexed forms as discussed above. Starting at pH ~6.3, resonances appear in the spectrum for the kinetically stable Zn(II)–GHL complexes, including a single resonance at 8.77 ppm

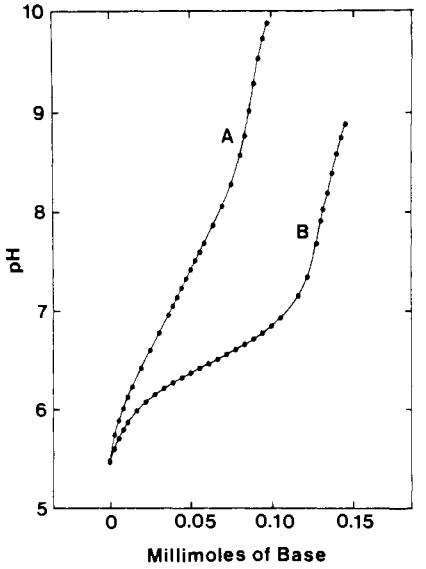


Figure 7. Typical potentiometric titration curves: (A) 0.043 mmol GHL; (B) 0.043 mmol GHG and 0.042 mmol $Zn(NO_3)_2$. Half of the experimental points are shown. The solid curves through the experimental points are theoretical titration curves calculated with the pK_A values for GHL reported in the text and the formation constants for the Zn(II)–GHL complexes shown in Table II.

for Lys NH of complexed GHL. At pH 7.21, the exchange-averaged resonances for the free and kinetically labile complexes have completely disappeared, and the resonances for the kinetically stable complexes are the only resonances observed. The pH dependence of the various resonances observed for GHL in the presence of Zn(II) is shown in Figure 6. The integrated intensity of the Lys NH resonance is the same as that of the C2-H and C4-H resonances assigned to complex **2** over the pH range 6.3–7.8, indicating that the Lys NH resonance is also for complex **2**. The absence of a Lys NH resonance for complex **1** suggests either that it has been displaced by Zn(II) or that it exchanges faster with solvent protons than do the Lys NH protons of free GHL and complex **2**.

Formation constants for the Zn(II)–GHL system were determined from pH titration data. Solutions containing GHL and GHL + Zn(II) were titrated with standardized KOH; representative titration data are shown in Figure 7. The acid dissociation constants determined for GHL are $pK_1 = 2.91$, $pK_2 = 6.51$, $pK_3 = 7.88$ and $pK_4 = 10.48$, with standard deviations of 0.01 pK unit or less. Preliminary analysis of the titration data for the Zn(II)–GHL solutions indicated titration of more protons than are titrated from GHL alone. For solutions having GHL:Zn(II) ratios of 1:1 or larger, an average of 0.96 mmol of additional protons were titrated per mole of Zn(II). Since the ammonium group of the lysine residue has a pK_A of 10.48, and the ^1H NMR data shows it to be protonated in the Zn(II)–GHL complexes, this group was not considered in the proton balance or formation constant calculations.

Formation constants, defined by eq 1 and 2, were evaluated from titration data starting at pH 4 up to a maximum of pH 9.5,

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

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

where L is GHL from which carboxylic acid, imidazolium, and N-terminal ammonium protons have been titrated and the lysine amino group is protonated. Formation constants were calculated with the program MINIQUAD81. The zinc–hydroxy complexes $Zn(OH)^+$ ($\log \beta_1 = 6.62$) and $Zn(OH)_2$ ($\log \beta_2 = 11.44$) were included in the model. Although the Zn(II)–GHL solutions contained acetate ion, Zn(II)–acetate complexes were not included in the model because their formation constants are small and ^1H chemical shift data for the acetate resonance in the spectra of

Table II. Formation Constants of Zn(II)–Glycylhistidyllysine Complexes[a]

	$\log \beta_{11-1}$	$\log \beta_{110}$	$\log \beta_{111}$	$\log \beta_{12-1}$	$\log \beta_{120}$	$\log \beta_{11-2}$
Zn(II)–GHL[a,b]	−2.3	3.1	9.3	0.5	8.0	−11.8
Zn(II)–GHG[c]	−2.55	2.90				−12.24

[a] In 0.30 *m* KNO_3 at 25 °C. [b] Values given are the weighted averages of values determined from six independent experiments. The weighted standard deviations are ±0.1 or less. [c] In 0.15 M KNO_3 at 37 °C. Reference 16.

Zn(II)–GHL solutions indicate no detectable complexation by Zn(II). Binuclear species of the type $Zn_2(peptide)_x$ were repeatedly rejected by MINIQUAD81 and were eliminated from the model. The formation constants calculated from the titration data are given in Table II. Also listed in Table II are the formation constants determined by Agarwal and Perrin for the Zn(II)–GHG system.[16] The model used by Agarwal and Perrin is simpler, but includes the complexes found to be the most abundant in the Zn(II)–GHL system.

The formation constants in Table II can be combined to give acidity constants for the complexed ligand, as defined by eq 3–6.

$$ZnL \rightleftarrows ZnLH_{-1} + H^+ \quad (3)$$

$$K_{A1} = \frac{(ZnLH_{-1})(H^+)}{(ZnL)} \quad (4)$$

$$ZnLH_{-1} \rightleftarrows ZnLH_{-2} + H^+ \quad (5)$$

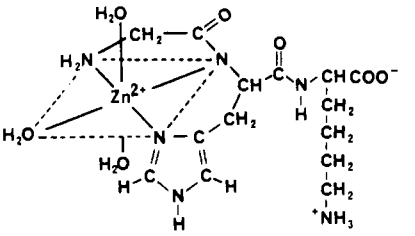
$$K_{A2} = \frac{(ZnLH_{-2})(H^+)}{(ZnLH_{-1})} \quad (6)$$

For the Zn(II)–GHL system, $pK_{A1} = 5.4$ and $pK_{A2} = 9.5$, while for the Zn(II)–GHG system, $pK_{A1} = 5.45$ and $pK_{A2} = 9.69$. The similarity of these constants as well as the formation constants in Table II provides additional evidence that the binding of Zn(II) by GHL and GHG is the same.

^1H NMR spectra were measured for the Zn(II)–GHL system in 99% H_2O/1% D_2O at the same concentrations used in the formation constant determinations. Fractional concentrations of GHL in the free and complexed forms were determined from the relative intensities of the resonances for the imidazole C2-H and C4-H protons. For comparison, species distributions were also simulated using the pK_A's and formation constants determined for the Zn(II)–GHL system. The sum of the fractional concentrations calculated for species having $r = -1$ is the same, within experimental error, as the fractional concentrations obtained from the sum of the intensities of the resonances for complexes **1** and **2**. The average of the absolute difference was 8.1%, while the estimated uncertainty of the fractional concentrations determined by NMR is 10%. The major complex with $r = -1$ in the computer simulated species distribution is $ZnLH_{-1}$, indicating that the kinetically stable Zn(II)–GHL complexes are of composition $ZnLH_{-1}$.

It is of interest now to consider the nature of the $ZnLH_{-1}$ complexes. The H_{-1} indicates that an extra proton has been titrated from the complex. Zn(II) also forms complexes of stoichiometry $ZnLH_{-1}$ with glycylhistidine and alanylhistidine.[14-17] The formation constants of the Zn(II)–glycylhistidine and Zn(II)–alanylhistidine complexes ($\log \beta_{11-1} = -2.14$ and −3.6, respectively)[14] are similar to $\log \beta_{11-1}$ of the Zn(II)–GHL and Zn(II)–GHG complexes. In the Zn(II)–glycylhistidine and Zn(II)–alanylhistidine complexes, the amide proton of the peptide bond is the extra proton titrated and the ligand is tridentate, with the Zn(II) binding to the N-terminal amino nitrogen, the deprotonated amide nitrogen, and the 1-imidazole nitrogen. The similarity of the formation constants suggests that the additional protons titrated to give the GHL and GHG complexes also are amide protons. Other possibilities are titration of a proton from the pyrrole group of the Zn(II)-coordinated imidazole side chain or from a coordinated water molecule. Since pK_A for Zn(II)-induced pyrrole ionization is estimated to be about 13,[19] as compared to pK_{A1} values of 5.4 and 5.45 for the Zn(II) complexes of GHL and GHG, respectively, it is unlikely that the extra proton is titrated from the pyrrole group. The pK_A value for titration of a proton from a coordinated water molecule in the Zn(II) complexes of nitrilotriacetic acid and β,β′,β″-triaminotriethylamine (10.0 and 11.1, respectively)[18] also suggest that the additional proton titrated in the formation of the $ZnLH_{-1}$ complex is not from a coordinated water molecule. However, their magnitudes suggest that the proton titrated from the $ZnLH_{-1}$ complexes of GHL and GHG to give the $ZnLH_{-2}$ complexes ($pK_{A2} = 9.5$ and 9.69, respectively) might be from a coordinated water molecule.

The similarity of the formation constants for the $ZnLH_{-1}$ complexes of GHL and GHG indicates that the bonding in the $ZnLH_{-1}$ complexes of the two tripeptides is the same. The NMR results are in agreement, since two kinetically stable complexes are formed with both tripeptides and the chemical shifts of their imidazole protons are essentially identical. Also, the chemical shift data for the ε-CH_2 protons of the lysine side chain indicate the lysine amino group is protonated and thus not involved in the binding in the GHL complex. The chemical shifts of the imidazole C2-H and C4-H resonances for complex **2** of both GHL and GHG are essentially identical with those of the $ZnLH_{-1}$ complexes of glycylhistidine and alanylhistidine,[14] leading to the conclusion that the binding in complex **2** of both GHL and GHG is tridentate, with Zn(II) coordinated to the glycylamino nitrogen, the deprotonated amide nitrogen of the glycylhistidyl peptide group, and the imidazole 1-nitrogen:



The nature of the Zn(II) binding in complex **1** cannot be established from the present data; however, the absence of a resonance for the histidyllysine peptide proton may indicate binding to a deprotonated amide nitrogen of the histidyl–lysine peptide bond. The chemical shift of the Gly CH_2 resonance is similar to that of the Gly CH_2 resonance of the Zn(II)–glycylhistidine complex and quite different from that of GHL with the amino group protonated, suggesting that the glycyl amino group is coordinated. However, the chemical shift of the Lys ε-CH_2 resonance is similar to that when the amino group of the lysine residue is protonated, indicating that the lysine amino group is protonated in complex **1**.

The structure shown above for complex **2** is identical with that of the Cu(II)–GHL complex,[7] which is thought to be a biologically active form of GHL. GHL also acts synergistically with Zn(II) to exert some biological activity.[5] It has been suggested that GHL might exert its functions by mediating the uptake of Cu(II) and possibly other transition metals into cells.[3] In the postulated model, the glycyl and histidyl residues of GHL are involved in Cu(II)-binding while the lysine side chain is necessary for recognition of a cell surface receptor. The results of this study show that Zn(II) forms an identical complex with GHL. However, the Zn(II) complex is of lower stability at physiological pH than the Cu(II) complex, which forms at pH ~4. This lower stability plus the distribution of Zn(II)–GHL between two complexes might account for the smaller effect of the Zn(II)–GHL system as compared to that of the Cu(II)–GHL system on cell growth if this complex is the biologically active species in the Zn(II)–GHL system.

Registry No. **2** (GHL), 98720-61-7; **2** (GHG), 98720-62-8.

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