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Determination of the Microscopic and Macroscopic Acid Dissociation Constants of Glycyl-L-histidyl-L-lysine and Related Histidine Peptides†

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ABSTRACT: Proton magnetic resonance studies of the acid-base chemistry of the glycyl ammonium, histidyl imidazolium, and lysyl ammonium groups of glycyl-L-histidyl-L-lysine and of the glycyl ammonium and histidyl imidazolium groups of glycyl-L-histidine and glycyl-L-histidylglycine are described. Chemical-shift data indicate that, at the molecular level, the glycyl ammonium and the histidyl imidazolium groups are titrated over the same pH range, with the acidity of the imidazolium group some 8 to 10 times that of the glycyl ammonium group, depending on the peptide. The lysyl ammonium group of Gly-His-Lys is much less acidic and is titrated over a higher pH range. Microscopic and macroscopic acid-dissociation constants were determined from chemical-shift data for each of the peptides. It is shown how microscopic formation constants for protonated metal complexes of these ligands, which are being used increasingly as models for the binding of metal ions by proteins, can be calculated from the macroscopic formation constants and the microscopic acid-dissociation constants. The acid-base chemistry of Gly-His-Lys is discussed with respect to its recently discovered biological activity.

In this paper, we characterize the acid-base chemistry of glycyl-L-histidyl-L-lysine and of the related peptides glycyl-L-histidine and glycyl-L-histidylglycine at the molecular level by nuclear magnetic resonance spectroscopy. Both microscopic and macroscopic acid-dissociation constants have been derived from chemical-shift measurements, and the distributions among the various protonated forms have been determined from the microscopic acid-dissociation constants.

The microscopic acid-base chemistry of these peptides is of interest in view of the use of histidine-containing peptides as model systems for the binding of metal ions by the histidyl residues of proteins (Agarwal and Perrin, 1975a,b; Aiba et al., 1974; Yokoyama et al., 1974). For example, recent studies of the copper(II) and cobalt(II) complexes of Gly-His and Gly-His-Gly in aqueous solution have indicated the presence of complexes in which either the amino nitrogen is protonated while the imidazole-3-nitrogen is metal complexed or the reverse (Agarwal and Perrin, 1975a,b). With the microscopic acid-dissociation constants reported in this paper, microscopic formation constants can be derived for the protonated complexes; such constants should provide better models for the binding of metal ions by the histidyl residues of proteins.

The microscopic acid-base chemistry of Gly-His-Lys is of interest in view of the report by Pickart and Thaler that a factor isolated from human serum prolongs the survival of normal rat liver cells and enhances the growth of hepatoma cells in culture (Pickart and Thaler, 1973). An amino acid analysis indicated the active factor to be a tripeptide formed by glycine, histidine, and lysine, and the synthetic tripeptide Gly-His-Lys was found to have biological properties similar to those of the native factor (Pickart et al., 1973). Pickart and Thaler suggested that the mechanism of action of the native factor and the synthetic tripeptide may reside in the high affinity of their polar side chains for DNA. The specific interaction of the imidazole moiety of histidyl residues of histidinamide and of small, synthetic histidine-containing oligopeptides with DNA is thought to be dependent on the protonation state of the interacting groups of the oligopeptide (Fritzsche, 1972; Goufevitch et al., 1974). The microscopic constants and the distribution between the protonation tautomers described in this paper should be of use in interpreting DNA binding experiments with Gly-His-Lys and similar peptides.

Experimental Section

Gly-His-Lys (Terochem Laboratories), Gly-His (Sigma) and Gly-His-Gly (Sigma) were used as received. pH measurements were made with an Orion 801 pH meter equipped with a Fisher microprobe combination electrode, which was calibrated with standard solutions of pH 4.00, 7.00, and 10.00. Acid-dissociation constants were evaluated both as mixed activity-concentration constants (activity of hydrogen ion and concentration of acid and its conjugate base) and as concentration constants. The acid-dissociation constants reported in Table I are mixed constants; factors for conversion to concentration constants are given in the footnote to Table I. pH measurements were converted to hydrogen ion concentration as described previously (Rabenstein and Sayer, 1976).

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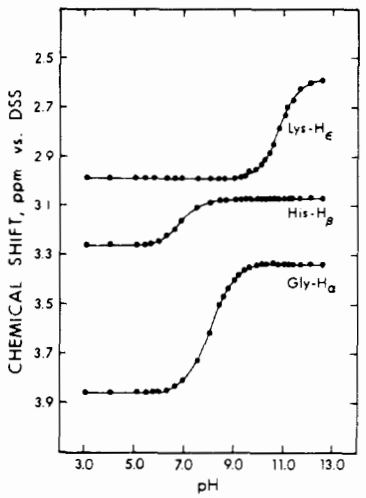

FIGURE 1: Chemical shifts of the protons on the ϵ carbon of the lysine residue (Lys-H_ϵ), the β carbon of the histidyl residue (His-H_β), and the α carbon of the glycyl residue (Gly-H_α) of Gly-L-His-L-Lys as a function of pH.

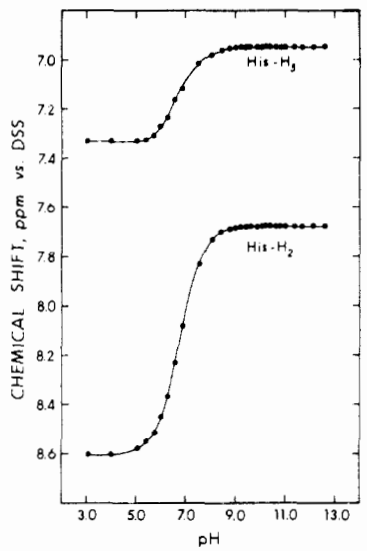

FIGURE 2: Chemical shifts of the protons on C-5 (His-H_5) and C-2 (His-H_2) of the imidazole ring of the histidyl residue of Gly-L-His-L-Lys as a function of pH.

NMR[1] spectra were obtained on Varian A60D or HA-100 high resolution spectrometers at 25 ± 1 °C. The probe temperature was checked periodically by lowering a thermometer or a thermocouple into the probe. Chemical shifts were measured with respect to the methyl resonance of internal *tert*-butyl alcohol or tetramethylammonium nitrate (TMAN) but are reported relative to the methyl resonance of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). Positive shifts correspond to protons less shielded than the methyl protons of DSS.

Solutions were prepared in distilled water and contained 0.035 M peptide, 0.080 M KCl, 0.01 M *tert*-butyl alcohol, and 0.01 M TMAN. For Gly-His and Gly-His-Gly, 40 mL of solution was prepared. The pH of each solution was adjusted initially to pH approximately 3 with concentrated HCl. NMR samples were then taken as the pH was increased by addition of concentrated KOH. Approximately 30 samples were taken over the pH range 3–13 for each peptide. Due to our having only a small amount of Gly-His-Lys, a single 3-mL Gly-His-Lys solution was used throughout the NMR titration experiment, and its pH was adjusted while in the NMR tube. The solution was placed in a 12-mm o.d. NMR tube and its pH adjusted to 2.97. After recording the NMR spectrum of the solution at this pH, the pH was adjusted by adding 1.5 M KOH with a syringe. The pH of the resulting solution was measured in the NMR tube with the microprobe pH electrode, and its NMR spectrum was then recorded. This procedure was repeated until the solution pH reached 12.45. Spectra were recorded at 29 pH values in the pH range 2.97 to 12.45. The ionic strength of the solution varied from 0.19 (pH 2.97) to 0.15 (pH 8.34) to 0.193 (pH 12.00) owing to changes in the concentrations of the partially deprotonated forms of the tripeptide.

The macroscopic and microscopic acid-dissociation constants were evaluated from chemical-shift data by the nonlinear least-squares curve-fitting procedures described previously (Rabenstein and Sayer, 1976). The curve-fitting calculations were done with the weighted nonlinear least-squares program KINET (Dye and Nicely, 1971).

Results

Glycyl-L-histidyl-L-lysine. Deprotonation of the acidic groups of Gly-His-Lys was monitored by observing the resonance patterns for the nonlabile protons on C-2 (His-H_2) and C-5 (His-H_5) of the imidazole ring and on the α, β, and ϵ carbons of the glycyl, histidyl, and lysine residues (Gly-H_α, His-H_β, and Lys-H_ϵ).[2] The resonances for His-H_2 and His-H_5 are multiplets up to pH 6.57 due to mutual coupling (Carlson and Brown, 1966); at pH greater than 6.57, both appear as singlets. The resonance pattern for His-H_β is a doublet over the entire pH range studied; that for Lys-H_ϵ is a broad multiplet at pH 2.97, probably due to coupling to the slowly exchanging protons of the adjacent ammonium group, which sharpens to a well-defined triplet at higher pH. The Gly-H_α protons are a singlet over the entire pH range studied.

The chemical shifts of these resonances are shown as a function of pH in Figures 1 and 2. The effect of deprotonation of acidic groups on the chemical shifts of carbon-bonded protons is attenuated rapidly by intervening chemical bonds; thus the chemical shift titration curves for His-H_2 and His-H_5 in Figure 2 are due almost entirely to titration of the imidazolium group while that for Gly-H_α in Figure 1 is due mainly to titration of the glycyl ammonium group.[3] The total changes in the chemical shifts of His-H_2 and Gly-H_α of Gly-His-Lys upon titration of both the glycyl ammonium and the histidyl imidazolium groups are 0.930 and 0.525 ppm, respectively. The change in chemical shift of His-H_2 of *N*-acetyl-L-histidine upon titration of the imidazolium group is 0.922 ppm, while that of the methylene resonance for the N-terminal glycyl residue of Gly-Gly-Gly is 0.513 ppm. Because of the number of bonds separating the Lys-H_ϵ protons from the imidazolium and glycyl ammonium groups, it is assumed that the titration

[1] Abbreviations used: His-H_2 and His-H_5 for the protons on C-2 and C-5 of the imidazole ring; Gly-H_α, His-H_β, and Lys-H_ϵ for the protons on the α, β, and ϵ carbons of glycyl, histidyl, and lysine residues; NMR, nuclear magnetic resonance; TMAN, tetramethylammonium nitrate; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

[2] In this paper, the imidazole ring of histidine is labeled according to IUPAC nomenclature. Thus, what is referred to as carbon-2, carbon-5, nitrogen-1, and nitrogen-3 of imidazole would be carbon-2, carbon-4, nitrogen-3, and nitrogen-1, respectively, in the biochemical nomenclature.

[3] The His-H_2 data are used in the calculation of microconstants because the effect of titration of the ammonium group on its chemical shift is less than the effect on the chemical shift of His-H_5 (Sachs et al., 1971).

TABLE I: Microscopic and Macroscopic Acid Dissociation Constants.[a-c]

	Gly-His-Lys	Gly-His	Gly-His-Gly
pK_2	6.60	6.77	6.62
pK_3	8.06	8.25	8.15
pK_4	10.71		
pk_{12}	7.54	7.88	7.62
pk_{13}	6.65	6.82	6.66
pk_{123}	7.10	7.14	7.16
pk_{132}	8.01	8.18	8.09

[a] Ionic strength varies from 0.15 to 0.19 M; 25 °C. [b] Mixed activity-concentration constants. To convert to concentration constants, subtract 0.10 from each pK or pk. [c] The linear estimate of the standard deviation, an indication of the goodness of fit, varies from 0.01 to 0.05 pK or pk units.

curve for Lys-H_ϵ is due entirely to titration of the lysine ammonium group.

The chemical shift results indicate that, as the pH is increased from 2.97, titration starts at the imidazolium group followed by the glycyl ammonium group and then, at somewhat higher pH, the lysine ammonium group is titrated. The titration curves for Gly-H_α and His-H_2 indicate some overlap of the pH regions over which the imidazolium and glycyl ammonium groups deprotonate, while those for Lys-H_ϵ and Gly-H_α indicate the glycyl ammonium group to be almost completely deprotonated before titration of the lysine ammonium group. Thus, deprotonation is predominantly by the microscheme shown in Figure 3, with a very small amount of deprotonation by a pathway involving deprotonation of the lysine ammonium group of III followed by deprotonation of the glycyl ammonium group. In Figure 3, the deprotonated imidazole ring is shown as the 1-H tautomer, by analogy with histidine (Reynolds et al., 1973). The acid-dissociation equilibrium to which a given microconstant refers is indicated by its subscript; the last number in the subscript denotes the group involved in the deprotonation step to which the constant applies while the preceding numbers denote groups titrated in preceding steps. The carboxylic acid, glycyl ammonium, imidazolium, and lysyl ammonium groups are labeled 1-4, respectively. The more familiar macroscopic stepwise acid-dissociation sequence is also shown in Figure 3; the relations between the macro- and microconstants are $K_2 = k_{12} + k_{13}$ and $K_3 = k_{123}k_{132}/(k_{123} + k_{132})$.

Macroconstants K_2, K_3, and K_4 were calculated from the chemical-shift titration curves for His-H_2, Gly-H_α, and Lys-H_ϵ by methods described previously (Rabenstein and Sayer, 1976). The procedure involved first calculating the fractional deprotonation of each group as a function of pH from the chemical-shift data. The fractional deprotonation of the glycyl ammonium and the imidazolium groups was calculated with eq 1 and 2

$$f_{2,d} = \frac{\delta_{g,obsd} - \delta_{g,p} - f_{3,d}\Delta_i}{\delta_{g,d} - \delta_{g,p} - \Delta_i} \quad (1)$$

$$f_{3,d} = \frac{\delta_{h,obsd} - \delta_{h,p} - f_{2,d}\Delta_a}{\delta_{h,d} - \delta_{h,p} - \Delta_a} \quad (2)$$

where $f_{2,d}$ and $f_{3,d}$ are the fractional deprotonations of the glycyl ammonium and the imidazolium groups, $\delta_{g,obsd}$ and $\delta_{h,obsd}$ are the chemical shifts observed for the Gly-H_α and His-H_2 resonances at the particular pH, $\delta_{g,p}$, $\delta_{g,d}$, $\delta_{h,p}$, and $\delta_{h,d}$ are the chemical shifts of the Gly-H_α and the His-H_2 protons for the nitrogen protonated and deprotonated forms of the

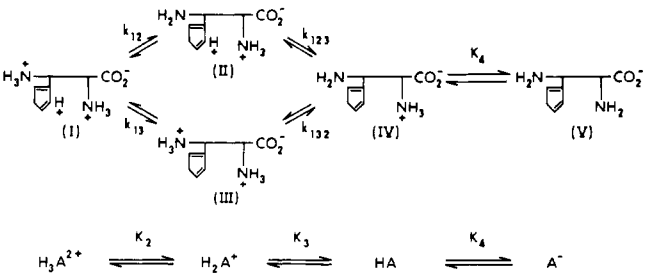

FIGURE 3: Microscopic deprotonation scheme for the imidazolium, glycyl ammonium, and lysyl ammonium groups of Gly-L-His-L-Lys.

molecule, Δ_i is the change in the chemical shift of Gly-H_α due to titration of the imidazolium group, and Δ_a is the change in the chemical shift of His-H_2 due to titration of the glycyl ammonium group. $f_{2,d}$ and $f_{3,d}$ were obtained simultaneously from the chemical-shift data for Gly-H_α and His-H_2 by first setting Δ_i and Δ_a to zero to obtain initial estimates for $f_{2,d}$ and $f_{3,d}$ at a particular pH and then iteratively refining these values. Using the chemical-shift data given above for model compounds, Δ_i was estimated to be 0.012 ppm and Δ_a 0.008 ppm. The fractional deprotonation of the lysine ammonium group, $f_{4,d}$, was calculated from the Lys-H_ϵ chemical-shift data with eq 3.

$$f_{4,d} = \frac{\delta_{1,obsd} - \delta_{1,p}}{\delta_{1,d} - \delta_{1,p}} \quad (3)$$

The average number of acidic protons per peptide molecule, $\bar{p}$, was then calculated as a function of pH from the fractional deprotonation values, according to eq 4

$$\bar{p} = 3 - f_{2,d} - f_{3,d} - f_{4,d} \quad (4)$$

$\bar{p}$ is plotted vs. pH in Figure 4. In terms of the protonated forms in the macroscopic titration sequence

$$\bar{p} = 3[H_3A^{2+}] + 2[H_2A^+] + [HA] \quad (5)$$

which leads to

$$\bar{p} = \frac{3a_{H^+}{}^3 + 2a_{H^+}{}^2K_2 + a_{H^+}K_2K_3}{a_{H^+}{}^3 + a_{H^+}{}^2K_2 + a_{H^+}K_2K_3 + K_2K_3K_4} \quad (6)$$

The values of pK_2, pK_3, and pK_4 in Table I were obtained by curve-fitting the data in Figure 4 to eq 6. The pH values at $\bar{p}$ = 2.5, 1.5, and 0.5 were used in the curve-fitting as initial estimates of pK_2, pK_3, and pK_4.

Microscopic constants for the imidazolium and glycyl ammonium groups were then obtained by curve-fitting the $f_{2,d}$ and $f_{3,d}$ values calculated above to eq 7 and 8

$$f_{2,d} = \frac{k_{12}a_{H^+} + k_{12}k_{123}}{a_{H^+}{}^2 + K_2a_{H^+} + K_2K_3} \quad (7)$$

$$f_{3,d} = \frac{k_{13}a_{H^+} + k_{13}k_{132}}{a_{H^+}{}^2 + K_2a_{H^+} + K_2K_3} \quad (8)$$

K_2 and K_3 were set equal to the values obtained from eq 6. Initial values for the microconstants for use in the curve-fitting calculations were obtained by extrapolation of plots of pM_x vs. $f_{x,d}$, where x = 2 or 3 and $pM_x = pH - \log [f_{x,d}/(1 - f_{x,d})]$ to $f_{x,d} = 0$ and 1 (Edsall et al., 1958). The pM_x plots are shown in Figure 5. The microconstants for Gly-His-Lys are given in Table I. It should be noted that a variation in pM, which is essentially an apparent dissociation constant for the particular group at each pH, with fractional deprotonation indicates that the acidity of the group is dependent on the protonation state of the other group.

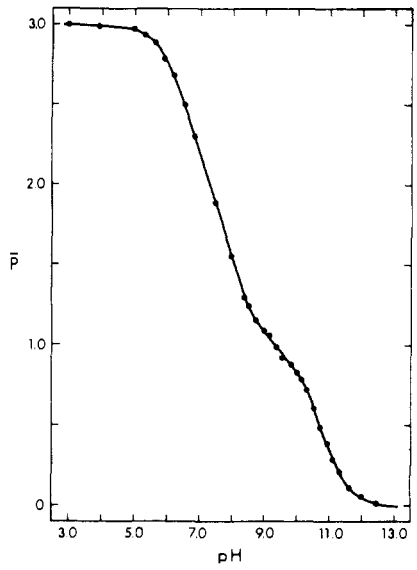

FIGURE 4: Average number of protons per peptide molecule, $\bar{p}$, vs. pH for Gly-L-His-L-Lys.

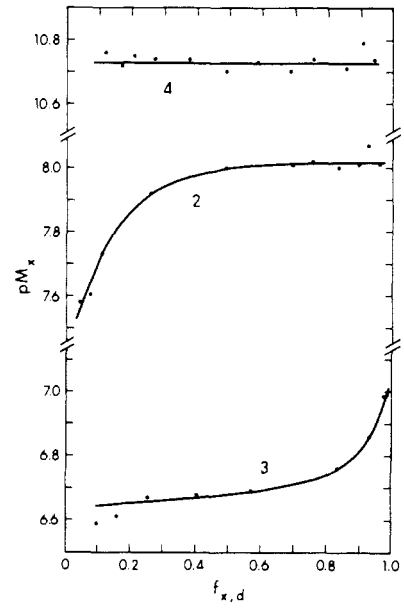

FIGURE 5: Apparent acid dissociation constant vs. fractional deprotonation for the glycyl ammonium (2), the histidyl imidazolium (3), and the lysyl ammonium groups (4) of Gly-L-His-L-Lys.

Uncertainty in the estimates used for Δ_i and Δ_a results in uncertainty in the fractional deprotonation values, and thus in the microconstants. The effect on the microconstants is small, however, with the microconstants for the minor pathway (pk_{12}, pk_{123} in Figure 3) being most sensitive to the uncertainty.[4]

Glycyl-L-histidine and Glycyl-L-histidylglycine. Chemical-shift titration curves were obtained for the Gly-H_α, His-H_2, and His-H_5 protons of Gly-His and Gly-His-Gly. For both peptides, the results are similar to those shown in Figures 2 and 3 for the same protons of Gly-His-Lys. The chemical-shift results indicate that, as the pH is increased from ~3, titration starts at the imidazolium group followed by the glycyl ammonium group with some overlap of the pH region over which the two groups are titrated. Thus, deprotonation of the imidazolium and glycyl ammonium groups proceeds by the microscheme shown in Figure 3 for the imidazolium and glycyl ammonium groups of Gly-His-Lys. Macroscopic and microscopic constants were determined for each peptide from the chemical-shift data for its Gly-H_α and His-H_2 protons by the procedure described above; the results are given in Table I. Values of $\Delta_i = 0.020$ ppm and $\Delta_a = 0.012$ ppm, for use in the calculation of $f_{2,d}$ and $f_{3,d}$ according to eq 1 and 2, were obtained from model compound data in the same manner as described above for Gly-His-Lys. The microconstants depend on the values used for Δ_i and Δ_a; however, as for Gly-His-Lys, the dependence is relatively small.[4]

Discussion

It is well known that the imidazolium and α-ammonium groups of peptides and proteins titrate over the same pH range (Nozaki and Tanford, 1967). The overlap of the pH ranges of the His-H_2 and Gly-H_α titration curves for Gly-His-Lys in Figures 1 and 2 and the corresponding curves for Gly-His and Gly-His-Gly indicate this to be the case for these peptides. However, macroscopic constant K_2 of Gly-His and Gly-His-Gly has generally been assigned to the imidazolium group and K_3 to the ammonium group (Agarwal and Perrin, 1975a; Yokoyama et al., 1974; Martin and Edsall, 1960). The results in Table I indicate this to be nearly correct, with from 89 to 92% of the titration of the imidazolium and glycyl ammonium groups of these peptides proceeding via the sequence I → III → IV (Figure 3). However, to characterize their chemistry at the molecular level, it is necessary to know the distribution between the microscopic forms.

Previous reports of NMR studies of the acid–base chemistry of Gly-His-Gly have concluded that the acidity of the imidazolium group is independent of the protonation state of the ammonium group (Markley, 1973, 1975). The mutual dependence of the pM values for the Gly-His-Gly imidazolium and ammonium groups on the protonation state of the other group indicates this not to be the case.

Microscopic Metal Complex Formation Constants. As mentioned in the introductory section, there is increasing interest in the use of histidine-containing peptides as models for the binding of metal ions by the histidyl residues of proteins. Such peptides contain several potential metal binding sites which, because of the structure of the peptide, are not likely to be simultaneously coordinated to the metal ion. Depending on the solution pH, the noncomplexed donor groups may be protonated. The formation constants which have been reported for such protonated complexes are macroscopic constants, expressed in terms of the sum of the concentrations of the tautomers of the particular protonation state. To illustrate, Agarwal and Perrin (1975b) reported the logarithm of the formation constant of the Co(II) complex of the monoprotonated form of Gly-His, as defined by eq 9, to be 2.23.

$$K = \frac{[CoHL^{2+}]}{[Co^{2+}][HL]} \qquad (9)$$

where [HL] is the sum of the concentrations of the two monoprotonated tautomers of Gly-His. Either the imidazole-3-nitrogen or the amino nitrogen could be metal coordinated, with the other nitrogen atom protonated (Agarwal and Perrin, 1975b). Attempts to determine which functional groups are metal complexed by comparison of the macroscopic formation constant with formation constants of Co(II) complexes of

[4] For example, for Gly-His-Lys pk_{13} and pk_{132} differ by $< \pm 0.02$ pk unit while pk_{12} and pk_{123} differ by $< \pm 0.10$ from the values listed in Table I when Δ_i and Δ_a are varied from 0 to twice the values used in calculating the microconstants in Table I.

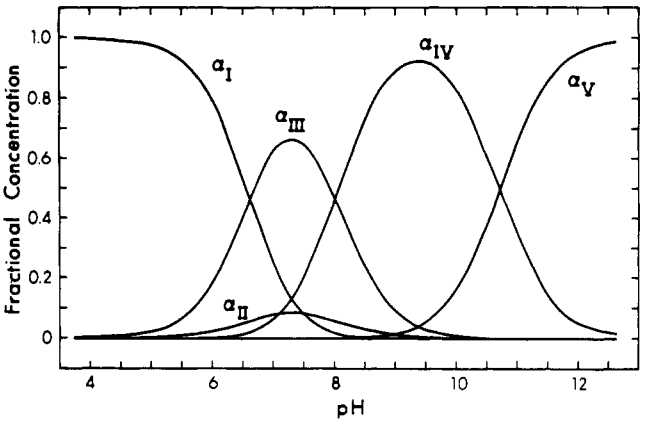

FIGURE 6: pH dependence of the fractional concentrations of the various protonated forms of Gly-L-His-L-Lys. α represents the fractional concentration of the species indicated by its subscript. See Figure 3 for identification of the species.

model ligands, e.g., *N*-acetyl-L-histidine, are ambiguous; the model compound formation constants refer to reaction with a specific form of the ligand whereas the macroscopic formation constant for CoHL is in terms of the sum of the concentrations of the monoprotonated tautomer, even though only one form may complex the metal. With the microconstants reported in Table I, the macroscopic formation constants can be converted to microscopic constants which can be more meaningfully compared with the formation constants of model complexes. For example, if in CoHL the imidazole-3-nitrogen is Co(II) coordinated and the amino nitrogen protonated, 0.89 ($= k_{13}/(k_{12} + k_{13})$) of the monoprotonated ligand is in the form which complexes the Co(II), and the logarithm of the microscopic formation constant for this complex is calculated to be 2.28. However, if the amino nitrogen atom is Co(II) coordinated in CoHL while the imidazole-3-nitrogen is protonated, 0.11 of the monoprotonated ligand is in the form which is in equilibrium with the complex and the logarithm of the microscopic formation constant is calculated to be 3.11. Comparison of these microscopic constants with the formation constants of the Co(II) complexes of *N*-acetylhistidine (2.35) (Martin and Edsall, 1960) and Gly-Gly (3.08) (Li et al., 1957) indicates that the previous conclusion of Agarwal and Perrin (1975b) that the imidazole-3-nitrogen is Co(II) coordinated in CoHL must be considered uncertain.

Glycyl-L-histidyl-L-lysine. The distribution of the various protonated forms of Gly-His-Lys as a function of pH is of interest in view of the possible dependence of its biological activity on its microscopic proton distribution (Pickart and Thaler, 1973; Pickart et al., 1973; Fritzsche, 1972; Goufevitch et al., 1974). The fractional concentrations of the microscopic forms, calculated from the microscopic acid-dissociation constants in Table I, are plotted as a function of pH in Figure 6. The quantity α represents the fractional concentration of the species indicated by the subscripts, which are identified in Figure 4. Goufevitch et al. (1974) proposed that the interaction of the imidazole moiety of histidine-containing oligopeptides with DNA is by hydrogen bonding of the imidazole >NH and >C=N- groups to >C=O and $-NH_2$ groups of two nucleic base pairs. These authors have also suggested that an electrostatic contribution to the stability of the complexes from interaction of adjacent basic amino acid residues, for example, lysyl or arginyl residues, with the negatively charged phosphate groups of DNA is essential. These considerations suggest that species III and IV, the most abundant forms of Gly-His-Lys in the physiological pH range, would have the greatest affinities for DNA.

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