A Reinterpretation of Paramagnetic Line Broadening in the Nuclear Magnetic Resonance Spectra of Amino Acids and Peptides. I. The Copper(II)–Glycine System[1]

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Abstract: Earlier interpretations of ^1H NMR line broadening effects in Cu(II) amino acid and peptide systems are challenged on three grounds: (i) the Cu(II)-containing entities involved in the line broadening have not been properly identified; (ii) exchange lifetime broadening has not been adequately distinguished from other relaxation effects; and (iii) the relative contributions of contact and dipolar interactions to the relaxation mechanism have not been established. In this study of the Cu(II)-glycine system we have used spectrophotometry to show that the species $CuHG^{2+}$, $CuHG_2^+$, and CuG_3^- exist in addition to CuG^+ and CuG_2 under conditions typical of the NMR experiments. We have developed a model to explain the line broadening results over the range $0 < pH < 11$ using the Swift and Connick theory for the effect of chemical exchange on NMR spectra. The model involves first-order ligand exchange with each of the species $CuHG^{2+}$, $CuHG_2^+$, and CuG_3^-, and second-order ligand exchange with the major species CuG_2. The system is found to be in "slow exchange" over several pH units, contrary to the general assumption of "fast exchange" in such systems. Measurements of T_1 and T_2 indicate that dipolar interactions make negligible contributions to the line broadening. Some results on the Cu(II)-sarcosine system are included to test inferences drawn from the glycine studies. We conclude that the ^1H NMR line broadening technique is not generally useful for obtaining structural information about such complexes in solution.

Several authors have claimed that it is possible to elucidate the Cu(II)-binding sites and structures of Cu(II) amino acid and peptide complexes using the selective broadening of the ligand ^1H NMR spectrum.[2] These claims are based on assumptions that: (i) Cu(II) is a "labile" ion and rapid ligand exchange transmits the effects of the Cu(II) ion from the bound ligand molecules to those in the bulk phase; and (ii) a dipolar relaxation mechanism is operating, allowing structural information about ligand molecules in the complex to be obtained from the r^{-6} dependence of the transverse relaxation times measured for the various ligand resonances. The complex species for which such structures have been proposed are typically species such as CuL or CuL_2 which are found in normal potentiometric titration studies.

The assumptions stated above are open to question. Amino acid and peptide complexes such as CuL and CuL_2 have high stability constants. It will be shown later that the lifetimes recorded for such complexes[3] are far too long to account for the observed magnitude of the line broadening at low Cu(II) concentrations. Moreover, the values of the transverse relaxation times (T_{2M}) of protons on coordinated ligands can be obtained from line broadening measurements only if the "fast exchange" condition is satisfied. This condition has not been verified experimentally in any of the antecedent NMR studies. Finally, even if the values of T_{2M} for the various relaxing species could be measured, no geometrical information about the bound ligand can be obtained unless it is shown that the dipolar interaction is dominant. The relative contributions of contact and dipolar interactions to the relaxation time of each proton have not been determined in previous line broadening studies.

In view of these doubts, we have reinvestigated the Cu(II)-glycine system. Visible spectroscopy was employed to establish the stoichiometries and stability constants of the species present under the conditions of the ^1H NMR line broadening measurements. The line broadening results were analyzed with the aid of the Swift and Connick theory[4] for the effects of chemical exchange on NMR spectra. T_1 and T_2 measurements were used to determine the relative contributions of the dipolar and contact interactions to the measured relaxation times.

Theory

The theory of the effects of paramagnetic ions on ^1H NMR spectra has been described elsewhere.[4-6] The following concepts and equations are essential for the present work.

The resonance absorption of a proton is characterized by the longitudinal relaxation time T_1, the transverse relaxation time T_2, and the chemical shift ω. The values of these parameters can be changed by a paramagnetic ion. Direct dipole-dipole coupling between the electron spin of the metal and the nuclear spin of the proton decreases with the distance r of the proton from the metal ion. Fermi contact interaction transfers unpaired spin density from the metal ion to the proton and is characterized by a coupling constant a.

The dipolar contributions to T_{1M}^{-1} and T_{2M}^{-1} are approximately equal.[7-9] The contact contributions can differ significantly depending upon the value of the electronic relaxation time. When T_{2M}^{-1} is considerably larger than T_{1M}^{-1}, it can be inferred that the relaxation mechanism is dominated by the contact term rather than the dipolar term.[9]

Chemical exchange of ligands between the coordination sphere and the bulk environment provides a mechanism for the transfer of the effects of the paramagnetic ion to the uncoordinated ligand molecules. A theoretical treatment of this process has been given by McConnell[10] and applied by Swift and Connick[4] to the specific case where complex ions (the paramagnetic environment, M) are present in low concentration compared with the free ligand molecules (the diamagnetic environment, L).[7]

The effect of a paramagnetic ion is to decrease T_2 from T_{2N} to T_{2L} and cause a corresponding increase in line width ($\pi\Delta\nu_{1/2} = T_2^{-1}$) defined as T_{2P}^{-1}.

$$T_{2P}^{-1} = T_{2L}^{-1} - T_{2N}^{-1} \quad (1)$$

The effect depends upon the type of relaxation mechanism and the rate of chemical exchange. When a ligand undergoes exchange between the paramagnetic metal ion environment and the diamagnetic free ligand environment, relaxation may occur by "$\Delta\omega$" or "T_{2M}" mechanisms.

These mechanisms involve changes in the precessional frequency ω and the transverse relaxation time of the ligand proton, respectively. For the limiting case of the T_{2M} mechanism alone, the general expression for T_{2P}^{-1} is:

$$T_{2P}^{-1} = \tau_L^{-1}\tau_M/(\tau_M + T_{2M}) \quad (2)$$

The concentration dependence of the exchange process depends upon the mechanism of the exchange reaction. For a first-order ligand exchange process,

$$ML_r \underset{k_r}{\overset{k_{-r}}{\rightleftharpoons}} ML_{r-1} + L \quad (3)$$

the lifetime τ_L of the ligand molecule in the bulk ligand environment and the lifetime τ_M of the ligand molecule in the metal ion coordination sphere are given by:

$$\tau_L = [L]_{total}/k_r[ML_{r-1}][L] \quad (4)$$

and

$$\tau_M = k_r^{-1} \quad (5)$$

For such a process, the measured relaxation time is directly proportional to the concentration of the relaxing species (ML_r).

For a second-order exchange reaction,

$$ML_r + {}^*L \underset{}{\overset{k}{\rightleftharpoons}} ML_{r-1}\,{}^*L + L \quad (6)$$

the lifetimes τ_L and τ_M are given by:

$$\tau_L = [L]_{total}/k[ML_r][L] \quad (7)$$

and

$$\tau_M = (k[L])^{-1} \quad (8)$$

If the system is in slow exchange ($\tau_M \gg T_{2M}$), T_{2P}^{-1} is proportional to the product of the concentration of the relaxing species (ML_r) and the free ligand concentration (L):

$$T_{2P}^{-1} = k[ML_r][L]/[L]_{total} \quad (9)$$

For the fast exchange limit ($\tau_M \ll T_{2M}$), T_{2P}^{-1} is simply proportional to the concentration of the relaxing species:

$$T_{2P}^{-1} = [ML_r]/[L]_{total} \cdot T_{2M} \quad (10)$$

Finally, the expression for T_{1P}^{-1} is similar to eq 2 because $\Delta\omega_M$ does not contribute to this relaxation mechanism.[9,11,12]

$$T_{1P}^{-1} = \tau_L^{-1}\tau_M/(\tau_M + T_{1M}) \quad (11)$$

Complex Equilibria

The complex equilibria present in solutions of amino acids or peptides containing Cu(II) ions are conveniently described by the general equation:[13]

$$pM^{z+} + qH^+ + rL^{y-} \rightleftharpoons M_pH_qL_r^{(zp+q-yr)+} \quad (12)$$

The corresponding overall stability constants are defined as:

$$\beta_{pqr} = [M_pH_qL_r]/[M]^p[H]^q[L]^r \quad (13)$$

For glycine, it is necessary to consider only forms of the ligand (G^-) with $q = 1$ or 2. In the present study, $p = 1$ because we are concerned with high ligand concentrations where $[L]_{total} \gg [M]_{total}$ and only monomeric species are important.[13] With $p = 1$, the mass balance equations for $[M]_{total}$ and $[L]_{total}$ yield:

$$[M] = \frac{[M]_{total}}{1 + \sum_q \sum_r \beta_{1qr}[H]^q[L]^r} \quad (14)$$

and

$$[L] = \frac{[L]_{total}}{1 + \sum_q \beta_{0q1}[H]^q} \quad (15)$$

Thus at any [H] it is possible to calculate the free ligand concentration [L] and subsequently the free metal concentration [M]. The validity of the above approximation has been tested by an iterative solution of the complete mass balance equations using the method of Ingri and Sillén.[14]

Spectrophotometric Determination of Stability Constants

The spectrophotometric data for the Cu(II)-glycine system required two different methods of analysis, depending on the pH range. At high pH, the system behaved as though only two major species, differing by one unit in r, were present. Such an equilibrium is described by eq 3 with a stepwise equilibrium constant

$$K_r = [CuH_qG_r][CuH_qG_{r-1}]^{-1}[G]^{-1} \quad (16)$$

Visible spectra recorded as functions of pH and $[G]_{total}$ should show an isosbestic point, and the system may then be analyzed by standard methods.[15]

At low pH, the spectrophotometric data for the Cu(II)-glycine system required the presence of major complex species which differed with respect to both q and r. The possible species are Cu^{2+}, $CuH_qG^{(1+q)+}$ ($q = 0, 1$), and $CuH_qG_2^{q+}$ ($q = 0, 1, 2$). The species with $q = 0$ have been well defined by potentiometric titration.[16,17] However, when the glycine concentration is high and $[G]_{total} \gg [Cu]_{total}$, additional species exist along with CuG^+ and CuG_2. We have characterized these additional species by extending a method previously used to obtain acid ionization constants from spectrophotometric data where overlapping equilibria exist.[18] Up to two additional species can be treated at a time.

Results and Discussion

Spectrophotometric Identification of Cu(II)-Containing Species. The visible absorption spectra of Cu(II)-glycine mixtures were recorded as functions of the pH at constant total ligand concentrations (Figures 1 and 3), and of the total ligand concentration at constant pH (Figure 5).

The absorbance-pH profile at 780 nm ($[G]_{total} = 1.0\ M$, $[Cu(II)]_{total} = 0.025\ M$) is presented in Figure 1. The dashed profile in Figure 1 was calculated on the assumption that the only Cu(II)-containing species were Cu^{2+}, CuG^+, and CuG_2.[19] The poor agreement between the observed and calculated profiles suggests that additional complex species are present.

In the range $0 < pH < 5$, species $CuHG^{2+}$, $CuH_2G_2^{2+}$, and $CuHG_2^+$ were considered in addition to Cu^{2+}, CuG^+, and CuG_2. The best agreement between the experimental and calculated profiles was obtained when only $CuHG^{2+}$ and $CuHG_2^+$ were included as major additional species. In this range there exist two pH's at which the absorbance has the same value. Beer's law and eq 14 lead to two simultaneous equations relating the absorbance to the β's and ϵ's of all the species. In the particular case of two complex species x and y with overall stability constants β_x, β_y and extinction coefficients ϵ_x, ϵ_y, these simultaneous equations may be solved for terms containing the unknowns $(\bar{\epsilon} - \epsilon_x)\beta_x$ and $(\bar{\epsilon} - \epsilon_y)\beta_y$, where $\bar{\epsilon} = (OD)_\lambda[Cu]_{total}^{-1}$ for a 1-cm cell. This leads to expressions of the form:

$$\bar{\epsilon} = \epsilon_x + X\beta_x^{-1} \quad (17)$$

$$\bar{\epsilon} = \epsilon_y + Y\beta_y^{-1} \quad (18)$$

The observable $\bar{\epsilon}$ is plotted vs. the calculated quantity X for several pairs of pH points. The slope and the intercept of

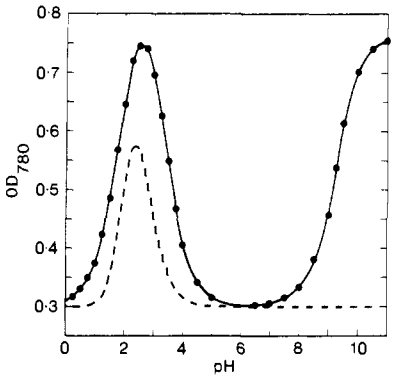

Figure 1. Dependence of the absorbance at 780 nm upon the measured pH in the Cu(II)-glycine system. Experimental points (●) were recorded with $[G]_{total} = 1.0\ M$, $[Cu(II)]_{total} = 1.5 \times 10^{-2}\ M$, $\mu = 2.0\ M$ (KNO_3), $T = 30°C$, solvent H_2O, and 1-cm path length. The dashed curve was calculated assuming that Cu^{2+}, CuG^+, and CuG_2 are the only Cu(II)-containing species. The solid curve was calculated with $CuHG^{2+}$, $CuHG_2^+$, and CuG_3^- as additional species, using the constants listed in Table I.

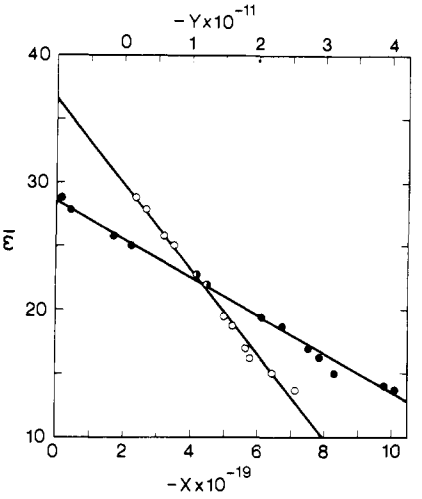

Figure 2. Graphs of $\bar{\epsilon}$ (l. mol^{-1} cm^{-1}) at 780 nm vs. X (○) and Y (●). The $\bar{\epsilon}$ values are from the data of Figure 1 in the range $0 < pH < 5$. The expressions for X and Y are given in footnote 20.

Table I. Values of Thermodynamic Constants for the Cu(II)–Glycine System

Quantity	Value	Ref
Log β_{011}	9.6[a] (H_2O)	17, this work
	9.8[a] (D_2O)	This work
$\Delta H(\beta_{011})$	−11 kcal mol^{-1} [b]	29
Log β_{021}	12.05[a]	17
$\Delta H(\beta_{021})$	−12 kcal mol^{-1} [b]	29
Log β_{101}	8.3[c]	17
$\Delta H(\beta_{101})$	−6 kcal mol^{-1} [b]	29
Log β_{102}	15.2[c]	17
$\Delta H(\beta_{102})$	−13 kcal mol^{-1} [b]	29
Log β_{111}	10.52 ± 0.04[c,d] (H_2O)	This work
Log β_{112}	18.48 ± 0.05[c,d] (H_2O)	This work
K_3	1.7 ± 0.4 M^{-1}[c,d] (H_2O)	This work
	1.9 ± 0.2 M^{-1}[c,d] (D_2O)	This work
	1.70 ± 0.03 M^{-1}[c,e] (H_2O)	This work
Log β_{103}	15.43 (= $K_3\beta_{102}$)	This work

[a] Potentiometric titration with glass electrode. [b] Calorimetric determination. [c] Spectrophotometric determination. [d] pH titration with constant total ligand concentration. [e] Total ligand concentration variation at constant pH.

the straight line plot yield β_x and ϵ_x, respectively. Similarly, a plot of $\bar{\epsilon}$ vs. Y yields β_y and ϵ_y. Deviations from linear relationships between $\bar{\epsilon}$ and X and Y indicate that the model is inadequate.

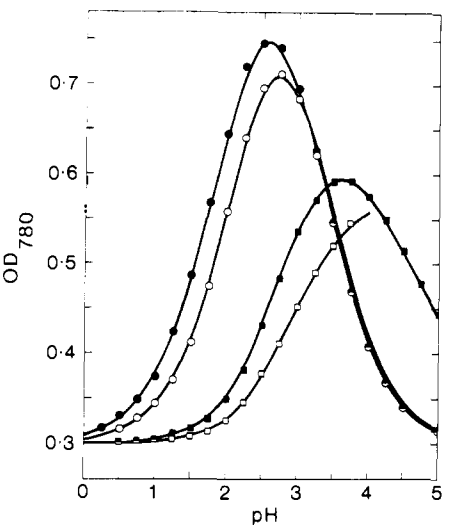

Figure 3. The dependence of the absorbance at 780 nm upon the measured pH at various total ligand concentrations. The $[G]_{total}$ values are 1.0 M (●), 0.5 M (○), $3.0 \times 10^{-2}\ M$ (■), and $1.5 \times 10^{-2}\ M$ (□). Other experimental conditions were identical with those of Figure 1.

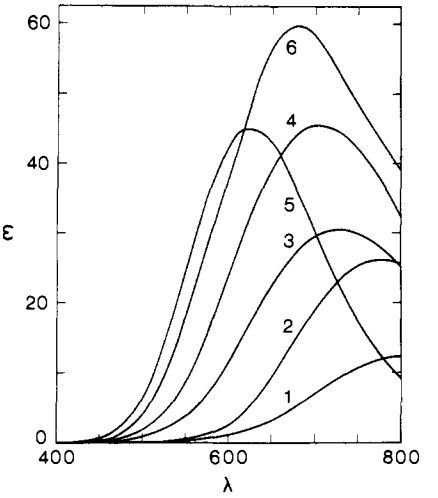

Figure 4. Absorption spectra (ϵ in l. mol^{-1} cm^{-1}) in the range $400 \leq \lambda \leq 800$ nm for the species Cu^{2+} (1), $CuHG^{2+}$ (2), CuG^+ (3), $CuHG_2^+$ (4), CuG_2 (5), and CuG_3^- (6). Curves 1 and 5 are taken from spectra recorded with solutions containing effectively only Cu^{2+} or CuG_2, respectively. The remaining curves are calculated from the data of Figures 3 and 5 and the constants listed in Table I.

Expressions for X and Y were derived for the above model which contains the species Cu^{2+}, CuG^+, CuG_2, $CuHG^{2+}$, and $CuHG_2^+$.[20] Graphs of $\bar{\epsilon}$ vs. X and $\bar{\epsilon}$ vs. Y are shown in Figure 2 The resulting values of β_{111} and β_{112} are included in Table I.

The observed and calculated absorbance-pH profiles for a range of total glycine concentrations are compared in Figure 3. The good agreement at all glycine concentrations confirms that the main species have been correctly identified. The calculated visible absorption spectra of the species $CuHG^{2+}$ and $CuHG_2^+$ in the range $400 \leq \lambda \leq 800$ nm are presented in Figure 4. There is good agreement between the λ_{max} values observed for $CuHG^{2+}$ (~790 nm) and $CuHG_2^+$ (700 nm), and the values (800 and 695 nm, respectively) which can be predicted[21] if HG is coordinated through its carboxyl group and G^- is chelated via its carboxyl and amino groups.

In the range $7 < pH < 11$, the absorption spectra record-

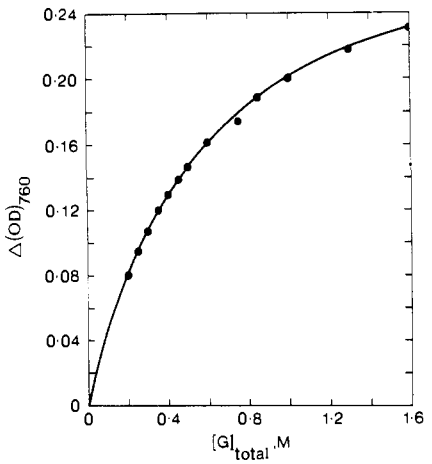

Figure 5. The dependence of the change in absorbance at 760 nm upon the total glycine concentration at a constant pH of 11.0. Experimental points (●) were recorded with solutions $[Cu(II)]_{total} = 1.025 \times 10^{-2}$ M, $\mu = 2.0$ M (KNO_3), $T = 30°C$, solvent H_2O. A solution containing effectively only the species CuG_2 was used as reference. Solid curve was constructed with constants obtained from the linear $\Delta(OD)_{760}^{-1}$ vs. $[G]_{total}^{-1}$ plot.

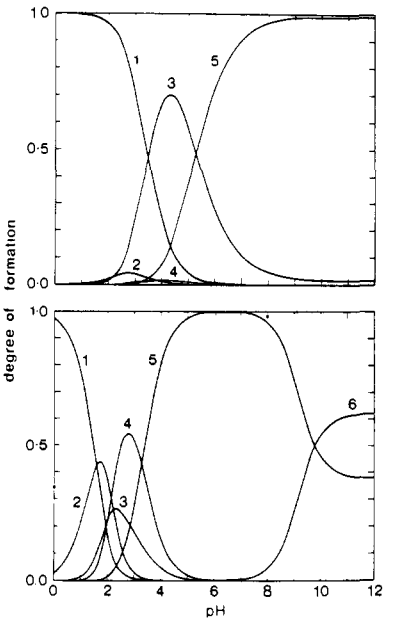

Figure 6. Species distribution curves for the Cu(II)-glycine system calculated with the stability constants from Table I. The degree of formation of each of the species Cu^{2+} (1), $CuHG^{2+}$ (2), CuG^{+} (3), $CuHG_2^{+}$ (4), CuG_2 (5), and CuG_3^{-} (6) is plotted as a function of pH for two total ligand concentrations: 8(a), $[G]_{total} = 1.0 \times 10^{-2}$ M; 8(b), $[G]_{total} = 1.0$ M. In both cases $[Cu(II)]_{total} = 5.0 \times 10^{-3}$ M.

ed at several pH values for $[G]_{total} = 1.0$ M and $[Cu]_{total} = 0.025$ M showed an isosbestic point at 615 nm. The major species present in this pH range is CuG_2, whose stability constant and extinction coefficients are known.[19] The monotonic absorbance-pH profile at 760 nm (cf. Figure 1) was treated as described above.[15] The additional species is identified as CuG_3^{-} since its concentration increases when the total ligand concentration is increased at constant pH (Figure 5). The values of K_3 calculated from the data in Figure 1 (constant ligand concentration, variable pH) and Figure 5 (constant pH, variable ligand concentration) are 1.7 ± 0.4 and 1.70 ± 0.03 M^{-1}, respectively. A similar value of K_3, 1.9 ± 0.2 M^{-1}, was obtained by repeating the experiments of Figure 1 in D_2O instead of H_2O. The visible spectrum of CuG_3^{-} is included in Figure 4.

Species distribution curves showing the degree of formation of each species as a function of the pH were calculated for two sets of conditions: $[G]_{total} = 1.0 \times 10^{-2}$ M (Figure 6a) and $[G]_{total} = 1.0$ M (Figure 6b). In both cases, $[Cu(II)]_{total} = 5.0 \times 10^{-3}$ M. The additional species $CuHG^{2+}$, $CuHG_2^{+}$, and CuG_3^{-} are in low concentration under conditions which are typical[17] of potentiometric titration experiments (Figure 6a). However, under conditions typical of NMR line broadening experiments (Figure 6b), these additional species predominate.

Previous ^1H NMR line broadening studies of Cu(II)-amino acid and Cu(II)-peptide systems[2,22-29] have been interpreted in terms of the species detected by the usual potentiometric titration procedures. The present work shows that extrapolation from potentiometric titrations in dilute solutions is not justified since additional species may be formed under the conditions of high ligand concentration which are used for the NMR experiments.

Interpretation of the pH-Dependent Line Broadening. The ^1H NMR spectrum of glycine in D_2O solution consists of a single resonance whose chemical shift ω_N is pH dependent.[30] The broadening of this resonance caused by the addition of Cu(II) ion to a 1.0 M glycine solution over the range $0 < pH < 11$ is shown in Figure 7. The broadening has been normalized by dividing $(\delta\nu_{1/2})_P$ by the total Cu(II) concentration. The dependence of the broadening upon the total Cu(II) concentration has been found to be linear in those systems where it has been examined.[2,22-24,31,32] We have confirmed the linearity at pH 11

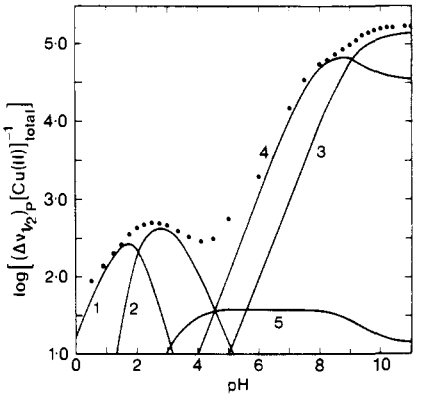

Figure 7. Dependence of $\log((\Delta\nu_{1/2})_P[Cu(II)]_{total}^{-1})$ upon the measured pH in the Cu(II)-glycine system. Experimental points (●) recorded at 100 MHz with $[G]_{total} = 1.0$ M, $\mu = 2.0$ M (KNO_3), $T = 30°C$, and D_2O solvent. Solid curves 1, 2, 3, and 5 represent the calculated contributions of first-order ligand exchange processes of the species $CuHG^{2+}$, $CuHG_2^{+}$, CuG_3^{-}, and CuG_2, respectively. Curve 4 is the calculated contribution of the second-order ligand exchange process of the species CuG_2. Curves were constructed with the constants listed in Tables I and III.

for the Cu(II)-glycine system over the range 2.5×10^{-6} $M \leq [Cu(II)]_{total} \leq 6.0 \times 10^{-5}$ M. Further, $(\Delta\nu_{1/2})_P$ is independent of the observation frequency (60 or 100 MHz), and no shift in the resonance of the free ligand was observed even at the highest Cu(II) concentrations that could be employed (6×10^{-5} M at pH 11). The absence of chemical shift effects is consistent with observations on other Cu(II)-containing systems.[32] We conclude that a $\Delta\omega$ mechanism in the fast exchange limit is not responsible for the observed line broadening. Consequently, eq 2 can be applied to this system.

Kinetics measurements on the Cu(II)-glycine system[3,33] have shown that the first-order dissociation rates k_{-r} of the species CuG^{+} and CuG_2 are of the order of 10 and 100

Table II. Summary of Literature Values for the Dissociation Rate Constant (k_r) and the Corresponding Lifetime (τ) for the Species CuG^+ and CuG_2

Species	k_r (sec^{-1})	τ (sec)	Ref
$Cu(Gly)^+$	34	2.9×10^{-2}	33
	23	4.3×10^{-2}	3
$Cu(Gly)_2$	50	2.0×10^{-2}	33
	120	8.3×10^{-3}	3

sec^{-1}, respectively (Table II). The residence times of glycine molecules in these species are therefore of the order of 10^{-1} and 10^{-2} sec, respectively. However, the data of Figure 7 at pH 11 lead to a calculated value of 1.9×10^{-6} sec for the term $(\tau_M + T_{2M})$. This value represents an upper limit of both the quantities τ_M and T_{2M} of the species responsible for the relaxation process. The complexes CuG^+ and CuG_2 have lifetimes which are three to four orders of magnitude greater than this calculated upper limit. Accordingly, *first-order* dissociation of CuG^+ and CuG_2 cannot be a major cause of the line broadening.

The Swift and Connick equation (2),[4] summed over all the Cu(II)-containing species known to exist in this system, was used in conjunction with the mass balance equations (14 and 15) to interpret the observed line broadening-pH profile (Figure 7). The general expression is:

$$\frac{[G]_{total}\,\pi(\Delta\nu_{1/2})_P}{[Cu(II)]_{total}} = T_{2O}^{-1} + \frac{\sum_q \sum_r (\tau_M + T_{2M})_{1qr}^{-1}\beta_{1qr}[H]^q[L]^r}{1 + \sum_q \sum_r \beta_{1qr}[H]^q[L]^r} \quad (19)$$

where the term T_{2O}^{-1} is a correction for bulk magnetic susceptibility and long-range dipolar interactions.[11]

The simplest model which we have been able to fit to the entire observed $(\Delta\nu_{1/2})_P$-pH profile, and which is compatible with the known equilibrium and kinetic data, involves the following major processes:

$$CuHG^{2+} \rightleftharpoons Cu^{2+} + HG \quad (I)$$

$$CuHG_2^{+} \rightleftharpoons CuG^+ + HG \quad (II)$$

$$CuG_3^- \rightleftharpoons CuG_2 + G^- \quad (III)$$

$$CuG_2 + {}^*G^- \overset{k}{\rightleftharpoons} CuG{}^*G + G^- \quad (IV)$$

The line broadening data at higher pH's (>5) are not adequately described by any combination of simple first-order ligand exchange processes (eq 3, steps I, II, and III). For example, if a value of $(\tau_M + T_{2M})$ for the species CuG_3^- is calculated from the experimental value of K_3 (1.7 M^{-1}, Table I) and the line broadening results at pH 11, the corresponding calculation of the broadening at pH 7 is over an order of magnitude too small. Similarly, if the model is constrained to fit the data at pH 7, the calculated broadening at pH 11 is much greater than that observed. Increasing K_3 by an order of magnitude only marginally improves the correspondence between the observed and calculated line broadening.

A second-order ligand exchange mechanism with CuG_2 (eq 6, step IV) in which τ_M is greater than T_{2M} for all pH values[26] is formally equivalent to a first-order exchange with CuG_3^- and suffers from the same difficulty as described above. A satisfactory fit to the data can be obtained only by introducing a second-order ligand exchange mechanism with CuG_2 in which $\tau_M > T_{2M}$ at pH values <9 and $\tau_M < T_{2M}$ at pH values >9 (cf. eq 8). That is, at pH <9 the lifetime of a ligand molecule in the CuG_2 coordination sphere determines the magnitude of the line broadening (eq 6, slow exchange). The line broadening is therefore directly proportional to the free ligand concentration at these pH values. However, at pH >9, τ_M becomes less than T_{2M} and does not influence the magnitude of the line broadening (eq 6, fast exchange). The broadening is then simply proportional to the concentration of CuG_2.

Table III. Values of Constants Used in the Line Broadening Model for the Cu(II)–Glycine System

Species	Quantity	Value
	T_{2O}	2.0×10^{-2} sec
$CuHG^{2+}$	$(\tau_M + T_{2M})$	5.3×10^{-4} sec
CuG^+	τ_M	4.4×10^{-2} sec[a]
$CuHG_2^+$	$(\tau_M + T_{2M})$	4.0×10^{-4} sec
CuG_2	τ_M	8.3×10^{-3} sec[a]
CuG_3^-	$(\tau_M + T_{2M})$	1.4×10^{-6} sec
CuG_2	T_{2M}	3.4×10^{-6} sec
CuG_2	k (eq 6)	$1.5 \times 10^7\ M^{-1}\ sec^{-1}$

[a] Taken from ref 3.

The concentration of CuG_2 decreases at high pH due to the formation of CuG_3^- and so the second-order process alone is not sufficient to explain the data. First-order exchange from CuG_3^- is required to make a contribution which increases as the pH is raised.

The contribution of each of these processes to the overall line broadening is shown in Figure 7. Also included (but making a smaller contribution) is the first-order ligand exchange reaction with CuG_2, calculated using a published value of τ_M.[3] The contribution of first-order ligand exchange with CuG^+ is several orders of magnitude smaller than the observed line broadening and is omitted from Figure 7. Values for the constants T_{2O},[34] $(\tau_M + T_{2M})_{1qr}$, $(T_{2M})_{102}$, and k used to calculate the component curves in Figure 7 are listed in Table III. The stability constants β_{1qr} are given in Table I. No attempt was made to refine the constants in Table III because the number of parameters involved in fitting the model to the experimental data is large.

Despite the complexity of this model, it fails to account entirely for the magnitude of the line broadening in the range $3.5 < pH < 6$. There are at least two possible explanations. Firstly, the published value[3] of τ_M which was used to calculate the contribution of first-order ligand exchange with CuG_2 may be too large. If the value were ca. 3×10^{-3} sec (instead of 8.3×10^{-3} sec), then a good fit to the data in the range $3.5 < pH < 6$ would be obtained. Secondly, a weak species with stoichiometry $CuHG_3$ may exist[35] and undergo a ligand exchange reaction

$$CuHG_3 \rightleftharpoons CuG_2 + HG \quad (20)$$

The distribution of $CuHG_3$ as a function of pH would be almost identical with that of CuG_2, except at high pH, where [HG] can no longer be treated as constant. The additional broadening from pH 3.5 to pH 6 could thus be caused by $CuHG_3$ instead of CuG_2.

The validity of the model was tested by comparing the experimental and calculated line broadening over a wide range of conditions. The observed and calculated dependences of $(\Delta\nu_{1/2})_P$ upon $[G]_{total}$ at pH 11 are shown in Figure 8. Similar good agreement was obtained at pH 2.5. The constants used in the calculations are listed in Tables I and III.

An essential feature of the proposed model is the "slow exchange" condition in the middle pH range. In order to test this condition further, we have extended the present study to the Cu(II)-sarcosine system in which the ligand has more than one proton resonance. We have confirmed

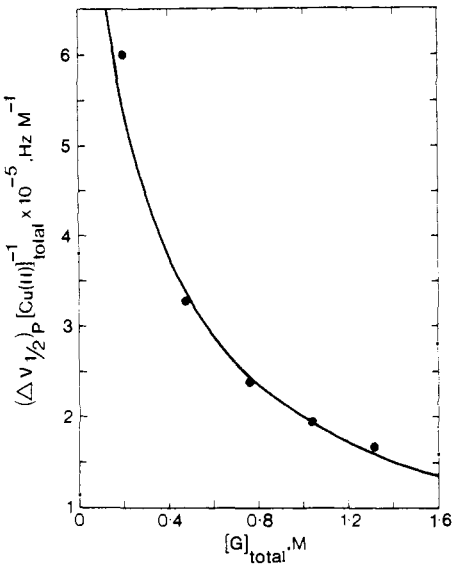

Figure 8. Plot of $(\Delta\nu_{1/2})_P[Cu(II)]_{total}^{-1}$ as a function of $[G]_{total}$ at pH 11.0. Experimental data (●) were recorded at 60 MHz with $[Cu(II)]_{total}$ in the range 1.0×10^{-5} to 5.0×10^{-5} M, $T = 30°C$, $\mu = 2.0$ M (KNO_3), and D_2O solvent. Solid curve was calculated with the constants from Tables I and III.

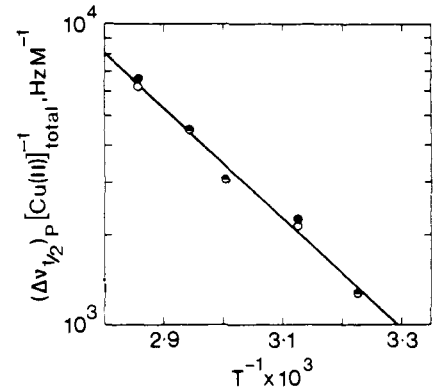

Figure 9. Plot of log $((\Delta\nu_{1/2})_P[Cu(II)]_{total}^{-1})$ as a function of temperature for the CH_2 (●) and CH_3 (○) resonances of sarcosine in the presence of Cu(II) ions at pH 7.0. Temperature range 37-77°C, $[Cu(II)]_{total} = 2.5 \times 10^{-3}$ M, total sarcosine concentration = 1.0 M with no KNO_3 added.

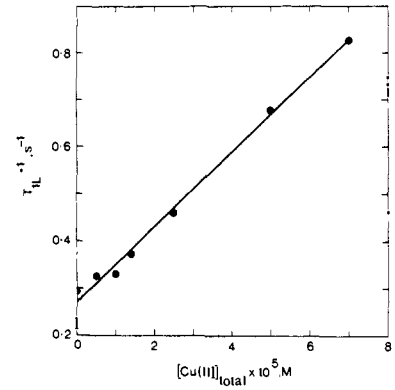

Figure 10. Dependence of the inverse longitudinal relaxation time of the glycine $-CH_2-$ protons, T_{1L}^{-1}, upon $[Cu]_{total}$. Each experimental point (●) was obtained by the 180°-τ-90° pulse sequence at 90 MHz. Conditions: $[G]_{total} = 1.0$ M, pH 11.0, $\mu = 2.0$ M (KNO_3), $T = 25°C$ and solvent D_2O.

that the Cu(II)-sarcosine and Cu(II)-glycine systems have absorbance-pH and $(\Delta\nu_{1/2})_P$-pH profiles which are qualitatively similar (even though they differ in quantitative respects). The sarcosine ^1H NMR spectrum consists of two uncoupled resonances due to the methylene protons and the $N-CH_3$ protons, respectively. At pH 11, these resonances are *differentially* broadened by Cu(II) ions (T_{2P}^{-1} (CH_3) $\simeq 3T_{2P}^{-1}$ (CH_2)). This observation is consistent with the two sets of protons having different T_{2M} values. At pH 7, the two sarcosine resonances are *equally* broadened by Cu(II) ions. This strongly suggests that the system is in "slow exchange" and that $\tau_M > T_{2M}$ (CH_3), T_{2M} (CH_2).

Temperature Dependence. The effect of temperature upon the equilibria in the Cu(II)-glycine system can be predicted using mass and charge balance relationships together with the constants[36] given in Table I. When the temperature of a sample of Cu(II)-glycine at pH 7 is raised, the pH decreases by approximately one unit over a 40°C range, whereas the concentrations of free ligand and the species CuG_2 remain unchanged. The same is true for the Cu(II)-sarcosine system at pH 7.[37]

In our line broadening model, at pH 7, the second-order exchange mechanism (reaction IV) dominates. Since [G] and $[CuG_2]$ remain constant as the temperature is raised:

$$T_{2P}^{-1} \propto k \qquad (21)$$

Thus, the line broadening is expected to increase with increasing temperature in the same manner as the rate constant k. This increase was confirmed by measurements on both the Cu(II)-glycine and Cu(II)-sarcosine systems. Furthermore, in the Cu(II)-sarcosine system the broadening of both resonances is the *same*, giving additional evidence that the system is in "slow exchange" over this temperature range. The experimental results for sarcosine are presented in Figure 9. The value of ΔH derived from the plot of log $((\Delta\nu_{1/2})_P[Cu(II)]^{-1})$ vs. T^{-1} is 8.5 ± 1.0 kcal mol^{-1}.

At pH 11 the line broadening decreases with increasing temperature in both the Cu(II)-glycine and sarcosine systems. This is again consistent with our model which predicts that the broadening is dominated by the species CuG_3^- at pH 11. When the temperature is raised, the stability constant K_3 decreases, the concentration of CuG_3^- falls, and the line broadening should be reduced.

T_1 Measurements. Even if the values of T_{2M} for the various species could be quantitatively determined, the deduction of structural information from these values requires the further condition that the contribution of the dipolar relaxation to T_{2M} predominates over the contact contribution. We have tested this condition for the Cu(II)-glycine system by performing T_1 measurements at pH 11. The results are presented in Figure 10. The value of $T_{1P}^{-1}[Cu(II)]_{total}^{-1}$ $(8 \pm 1) \times 10^3$ M^{-1} sec^{-1} compared with the value of $T_{2P}^{-1}[Cu(II)]_{total}^{-1}$ of $(4.9 \pm 0.3) \times 10^5$ M^{-1} sec^{-1} determined under identical conditions. Thus, $T_{2P}^{-1}:T_{1P}^{-1} \simeq 60$. This result is incompatible with a major contribution of the dipolar interaction to the observed line broadening. This conclusion has recently been confirmed independently in the cases of several Cu(II)-containing systems.[38]

Conclusion

Our treatment of line broadening in the Cu(II)-glycine system shows that it is not possible to obtain T_{2M} values *directly* from these data. Firstly, the system is in "slow exchange" ($\tau_M \gg T_{2M}$) over a wide pH range and τ_M makes an unknown contribution to the line broadening. Secondly, even at high pH, where dissociation of the species CuG_3^- is the predominant broadening mechanism, it is not certain whether the fast exchange condition ($\tau_M \ll T_{2M}$) applies. This could be determined by independent kinetic measure-

ments on the species CuG_3^-.[39] Only at the lowest pH's (<4), where the effects of carboxylate-bound molecules are being observed, can T_{2M} values be obtained in principle from the line broadening data. This is because τ_M values for such carboxylate-bound species are typically $\sim 10^{-8}$ sec,[32,40] whereas the measured values of $(\tau_M + T_{2M})$ are $\sim 10^{-3}$ sec. However, the complicated equilibria present at low pH in even this simple system cause difficulty in the quantitative determination of individual T_{2M} values. Such determinations are also subject to the general criticisms which apply to multiparameter curve-fitting procedures.

We conclude that there is a major difficulty in the measurement of T_{2M} values for Cu(II)-amino acid and Cu(II)-peptide complexes using the NMR line broadening approach. The difficulty is that of "time resolution" which applies to all forms of spectroscopy. In this case, the kinetic processes of ligand exchange are occurring on the same time scale as that of the detection method. Therefore it is in general not possible to obtain values of the intensive factors (T_{2M}) using only NMR line broadening measurements.

This argument could account for many of the difficulties which have been reported in determining binding sites in some systems.[2,23,41,42] For example, equal broadening of the imidazole proton resonances in systems such as Cu(II)-histidine and Cu(II)-carnosine does not necessarily imply that the Cu(II) ion is equidistant from both protons and therefore bound at the N3 site. Instead, at the pH's of these line broadening experiments, the system may be in "slow exchange" with the Cu(II) bound at the N1 site or at the N3 site.

Apart from the difficulty in obtaining T_{2M} values from NMR line broadening data, there are several other problems involved in the interpretation of such data. Firstly, most previous authors[2,22-29] have attempted to explain the observed line broadening in terms of the structures of the major stable species which have been detected by normal potentiometric studies on Cu(II)-amino acid and peptide systems. We have already commented on the doubtful validity of the assumption that all the species present at high ligand concentrations can be predicted from potentiometric measurements on much more dilute solutions. In addition, the results of a large number of kinetics studies show that these stable Cu(II)-amino acid and peptide complexes have ligand dissociation rates which are of the order of $10-10^3$ sec^{-1}.[3,33,43] Simple first-order dissociation of such complexes cannot be the major exchange mechanism responsible for the line broadening results at low Cu(II) concentrations.

To account for the line broadening reported in antecedent studies, it is possible to invoke either second-order ligand exchange mechanisms (cf. eq 6), or the existence of weak complexes which can undergo rapid ligand exchange. Both cases have been found in the Cu(II)-glycine system. In the former case, the "fast exchange" condition assumed in earlier studies is invalidated. In the latter case, any information gained from the line broadening data will have little relevance to the structures of the major species.

Secondly, as we have already indicated, in cases where T_1 and T_2 measurements have been performed[32,38] it has invariably been found that $T_{2P}^{-1} > T_{1P}^{-1}$. The r^{-6}-dependent dipolar term can then not be the predominant cause of relaxation. Thus it is unlikely that geometrical structural information can in general be obtained from such line broadening measurements, even if the species responsible for relaxation can be identified and the values of T_{2M} can be found.

Experimental Section

Materials. Glycine, sarcosine (Fluka, puriss.), KNO_3 (Riedel-de Haen A. G., A. R.), KOH (Merck, G. R.), and $Cu(NO_3)_2 \cdot 3H_2O$ (BDH, AnalaR) were used without further purification. The >99.8% D_2O solvent employed in these experiments was obtained from the Australian Atomic Energy Commission. The stock solution of $Cu(NO_3)_2$ used in all experiments was standardized by the $[Cu(en)_2][HgI_4]$gravimetric technique.[44]

NMR Measurements. NMR spectra were obtained on 0.5-ml samples in precision 5-mm tubes using Varian A-60, HA-100, XL-100, and JEOL PS-100 spectrometers operating in the continuous wave mode. The sample temperature was set to 30 ± 1°C using standard procedures.[45] Methanol and ethylene glycol were employed as standards for the calibration of temperature controllers. Measurements of the peak width at half peak height ($\Delta\nu_{1/2}$) were made from spectra recorded at either 50 or 100 Hz sweep width (Varian) or at 27, 54, or 108 Hz sweep width (JEOL). Care was taken to avoid saturation. Representative spectra were digitized and fitted to a Lorentzian line shape function. The values of $\Delta\nu_{1/2}$ from the best fit of the data to a Lorentzian line shape agreed with the values of $\Delta\nu_{1/2}$ obtained by direct measurements to within ±1%. The quantity $(\Delta\nu_{1/2})_P$ was found by subtracting the $(\Delta\nu_{1/2})_N$ for a particular glycine solution containing no Cu(II) from the $(\Delta\nu_{1/2})_L$ obtained with an identical glycine solution containing Cu(II) ions.

Measurements of T_1 for the glycine $-CH_2-$ resonance were performed on a Bruker WH-90 pulsed Fourier transform NMR spectrometer. Values of T_{1L} were obtained from linear plots of $\ln(1 - I_\tau/I_0)$ vs. τ, where I_0 and I_τ are the initial peak height and the peak height after a delay time τ, respectively.[46]

Visible Spectrophotometric Measurements. A Beckman Acta V uv-visible spectrophotometer was employed for all visible spectrophotometric measurements. A constant sample temperature of 30.0 ± 0.1°C was maintained with a Colora WK-5 thermostated water bath and jacketed cell holders.

pH Measurements and Titrations. Measurements of pH were made at 30.0 ± 0.1°C in a thermostated cell with a Radiometer PHM 4c meter, using Radiometer electrodes GK2320C (combined) or G202C (glass) and K401 (calomel). Electrodes were standardized against standard phthalate, phosphate, and borax buffers. The pH titrations and the determination of the pK_a of glycine were performed with a Radiometer TTT2 Titrator and ABU12 Autoburette. The titration vessel was thermostated to 30.0 ± 0.1°C. All pH values quoted refer to the actual pH meter readings at 30°C.

Treatment of Data. In general, where a nonlinear dependence of the measured quantity (e.g., OD or line broadening) upon some variable (pH, ligand concentration, etc.) was observed, the experimental data were replotted in a form which produced a linear dependence. Straight lines were fitted to the experimental data by linear least squares. Errors in derived quantities are quoted to the 99% confidence limit as determined by Student's t test. All experiments except the temperature dependence of the line broadening were repeated at least once.

Procedures. Stock solutions employed in these experiments were prepared in H_2O or D_2O by direct weighing of glycine, KNO_3, and KOH. Standardized solutions of HNO_3 and KOH in either H_2O or D_2O were used for pH adjustments. Cu(II) concentrations were adjusted by adding known volumes of standardized $Cu(NO_3)_2$ stock solution.

In experiments involving variation of the total Cu(II) concentration, two stock solutions were prepared with Cu(II) concentrations at each end of the range to be studied. The total ligand concentration, pH, and ionic strength were identical in both solutions. Calculated volumes of these two solutions were added together using a digital pipet (Digi-pet, 1 ml) in order to produce the desired Cu(II) concentration.

In order to record the visible and NMR spectra as functions of pH, pairs of solutions were prepared. The two solutions had identical total glycine concentrations, total Cu(II) concentrations, and ionic strengths, but had a high pH and a low pH, respectively. One solution was titrated into the other until the desired pH value was obtained. A spectrum was recorded. The procedure was repeated for each point. Cu(II)-free glycine solutions were titrated in the NMR experiments to enable the natural glycine line width at each pH point to be determined.

Solutions covering a range of total ligand concentrations were prepared from known volumes of two solutions whose total ligand

concentrations were at the extremes of the range. In both solutions the pH was the same and the ionic strength was maintained at 2.0 *M* with KNO_3. The Cu(II) concentration was the same for both extreme solutions in the spectrophotometric experiments, but was varied in the NMR experiments to maintain easily measurable line broadening (~5 Hz).

Potentiometric titrations of glycine in both H_2O and D_2O were carried out at 30°C to determine the operational values of β_{011} under conditions typical of the NMR and spectrophotometric experiments. The titrations were performed on 0.01 *M* glycine solutions with ionic strengths of 2.0 *M* (KNO_3). The average of two values of β_{011} are given in Table III for H_2O and D_2O.

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$$(20)\quad X = \frac{(\bar{\epsilon} - \epsilon_{CU})(H_2G_2^2 - H_1G_1^2)}{H_1H_2G_1G_2(G_1 - G_2)} + \frac{(\bar{\epsilon} - \epsilon_{101})\beta_{101}(H_2G_2 - H_1G_1)}{H_1H_2(G_1 - G_2)} + \frac{(\bar{\epsilon} - \epsilon_{102})\beta_{102}G_1G_2(H_2 - H_1)}{H_1H_2(G_1 - G_2)}$$

$$Y = \frac{(\bar{\epsilon} - \epsilon_{CU})(H_2G_2 - H_1G_1)}{H_1H_2G_1G_2(G_2 - G_1)} + \frac{(\bar{\epsilon} - \epsilon_{101})\beta_{101}(H_2 - H_1)}{H_1H_2(G_2 - G_1)} + \frac{(\bar{\epsilon} - \epsilon_{102})\beta_{102}(H_2G_1 - H_1G_2)}{H_1H_2(G_2 - G_1)}$$

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