N.m.r. and e.p.r. investigation of the interaction of copper(II) and glycyl-L-histidyl-L-lysine, a growth-modulating tripeptide from plasma

Jean-Pierre LAUSSAC,*† Raymond HARAN† and Bibudhendra SARKAR*‡

*The Research Institute, Hospital for Sick Children, Toronto, Ont. M5G 1X8, and Department of Biochemistry, University of Toronto, Toronto, Ont. M5S 1A8, Canada, and †Laboratoire de Chimie de Coordination, C.N.R.S., 31400 Toulouse, France

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Interaction of Cu(II) and Gly-His-Lys, a growth-modulating tripeptide from plasma, was investigated by ^{13}C - and ^1H -n.m.r. and e.p.r. spectroscopy. The n.m.r. line-broadening was interpreted in terms of major and minor species formed as a function of pH. The results indicate that the n.m.r. line-broadening is due to the presence of minor species in rapid exchange and not due to the major species in solution, which has a large τ_{M} . It is concluded that the technique of ^{13}C - and ^1H -n.m.r. line broadening, caused by paramagnetic Cu(II) ion, should be undertaken with caution, since the method may not be useful for obtaining structural information on the major species. The e.p.r. spectra over a wide pH range are almost entirely due to similarly co-ordinating species. Starting at pH 5.5, the narrowest absorption near 340 mT shows superhyperfine structure, which comes out sharply in the pH region 6.0–9.6. The spectra in this pH range showed the seven lines of nitrogen superhyperfine splitting, indicating clearly the co-ordination of three nitrogen atoms to Cu(II). The e.p.r. parameters in the medium pH range, $A_{\parallel} = 19.5\text{ mT}$ and $g_{\parallel} = 2.21$, fit well with the contention that Cu(II) is ligated to Gly-His-Lys through one oxygen atom and three nitrogen atoms in a square-planar configuration.

The tripeptide glycyl-L-histidyl-L-lysine has been co-isolated with copper and iron from human plasma in association with the albumin and α -globulin fractions at about 200 ng/ml (Pickart & Thaler, 1973, 1980; Schlesinger *et al.*, 1977; Pickart *et al.*, 1979). It has been proposed that the peptide acts synergistically with these transition metals to alter the growth and metabolism of cultured hepatoma cells and hepatocytes. There is also the evidence that the peptide forms complexes with the transition metals before it interacts with cells. Analyses of the solution equilibria of the Cu(II)–Gly-His-Lys system over a wide pH range indicated the presence of multiple species in solution (Lau & Sarkar, 1981). The results also showed that the tripeptide was able to compete with albumin for Cu(II) at physiological pH. The combined spectroscopic–potentiometric studies suggested the structure of a major complex species as the one that involves a carboxy group, one imidazole group and two other types of nitrogen atoms (Lau & Sarkar, 1981; Sarkar *et al.*, 1983). But an X-ray-crystallographic study of a Cu(II)–Gly-His-Lys complex

showed the existence of a tridentate bonding structure (Pickart *et al.*, 1980) involving the imidazole group, the α -amino group and a peptide backbone nitrogen atom, a structure similar to the one found for the Cu(II) complex of Gly-His (Blount *et al.*, 1967). However, the much higher value of the log (stability constant) of complex species MA ($\log \beta_{101} = 16.44$) in the Cu(II)–Gly-His-Lys system, as compared with the corresponding Cu(II) complexes formed by Gly-His and Gly-His-Gly [where M and A represent Cu(II) ion and anionic peptide respectively] ($\log \beta_{101} = 8.68$ and 8.52 respectively) (Agarwal & Perrin, 1975), raised the possibility of the involvement of an additional group in the co-ordination. The enhanced stability could be attributed to the involvement of either the carboxy oxygen atom and the imidazole, amino and peptide nitrogen atoms, or the carboxy oxygen atom and the α -amino, imidazole and ϵ -amino nitrogen atoms (Lau & Sarkar, 1981; Sarkar *et al.*, 1983). Such an assignment is consistent with a square-planar Cu(II) complex with an observed visible-absorption maximum at about 610 nm (Bryce & Gurd, 1966). Recently, further refinement of the X-ray structure

‡ To whom correspondence should be addressed.

of the same Cu(II)–Gly–His–Lys complex has shown that the carboxy oxygen atoms form bridges between Cu(II) centres, and thus the system is polymeric in the solid state (C. M. Perkins, N. J. Rose, R. E. Stenkamp, L. H. Jensen, B. Weinstein & L. Pickart, personal communication). However, these workers suggest that the polymers will not persist in dilute solution and that Gly–His–Lys will act as a tridentate ligand involving α -amino, peptide, and imidazole nitrogen atoms in the biological milieu.

In order to obtain a fuller description of the solution chemistry of the Cu(II)–Gly–His–Lys system, we have conducted ^{13}C - and ^1H -n.m.r. and e.p.r. investigations of the Cu(II)–Gly–His–Lys system over a wide range of pH. Results suggest a Cu(II)–Gly–His–Lys complex in solution that involves a carboxy group and three nitrogen atoms in a square-planar co-ordination.

Experimental

Materials

$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ was purchased from Fisher Scientific Co. (Ottawa, Ont., Canada), and sodium 4,4-dimethyl-4-silapentane-1-sulphonate was from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). $^2\text{H}_2\text{O}$ (99.8% ^2H) from the Centre d'Energie Atomique (Gif-sur-Yvette, France) was used as a solvent. The acetate form of glycyl-L-histidyl-L-lysine was obtained from Bachem (Bubendorf, Switzerland). The purity of the peptide was checked by t.l.c. with chloroform/methanol/aq. 17% NH_3 (2:2:1, by vol). The pH titrations were performed in $^2\text{H}_2\text{O}$ with either ^2HCl or NaOH . All pH values were measured on a Tucassel pH-meter calibrated with standard buffers (Merck Titrisol) and are quoted without correction for ^2H isotope effect. Stock solutions of CuCl_2 from $^1\text{H}_2\text{O}$ were prepared in 10mM- ^2HCl and calibrated titrimetrically. Copper(II) solution was added to the peptide solution with a Hamilton microlitre syringe to obtain corresponding ratios.

N.m.r. spectra

^{13}C -n.m.r. spectra were recorded at 22.63 MHz on a Bruker WH90 NMR spectrometer operating in the pulse–Fourier-transform mode and at 62.86 MHz on a Cameca 250 spectrometer. All spectra were recorded under conditions of full proton decoupling at an ambient probe temperature of $27 \pm 2^\circ\text{C}$ (22.63 MHz) or of $22 \pm 2^\circ\text{C}$ (62.86 MHz). Field stabilization was provided by an internal deuterium lock signal. Typical spectra required 4–10 h for Gly–His–Lys concentrations ranging from 0.1 to 0.3 M.

Spin–lattice relaxation-time (T_1 in seconds) measurements were performed by the inversion recovery technique (Freeman & Hill, 1971) by using

the $(180^\circ - \tau - 90^\circ - T_1)_n$ pulse sequence method, where τ is a variable time delay, $T_1 \geq 5(T_1)_{\text{max}}$, and $(T_1)_{\text{max}}$ is the longest longitudinal relaxation time to be measured. The value of the spin–lattice relaxation time is given by using a semi-logarithmic plot:

$$A = A_\infty [1 - 2 \exp(-\tau/T_1)] \quad (1)$$

where A is observed intensity and A_∞ is equilibrium intensity. The accuracy of T_1 values was $\pm 10\%$.

The ^1H -n.m.r. spectra were obtained on a Cameca 250 spectrometer equipped with a Nicolet B-NC 12 computer system operating at a probe temperature of 22°C . Variable temperature was measured to within $\pm 2^\circ\text{C}$, by using the OH chemical shift of methanol or of ethylene glycol. The system was locked on the internal $^2\text{H}_2\text{O}$. Peptide concentrations were approx. 10 mM. All chemical shifts (^{13}C and ^1H) were reported in p.p.m. downfield from internal 4,4-dimethyl-4-silapentane-1-sulphonate.

Paramagnetic relaxation by Cu(II)

The presentation and analysis of the n.m.r. results given below are based on theories and experimental procedures that have been extensively discussed in the literature (Swift & Connick, 1962; Luz & Meiboom, 1964).

The effects on the longitudinal (T_{1p}^{-1}) and transverse (T_{2p}^{-1}) relaxation rates of the observed nuclei due to the unpaired electron of a paramagnetic metal are obtained by subtracting the relaxation rate measured in the absence of the paramagnetic metal (T_{10}^{-1} , T_{20}^{-1}), from the rate observed in the presence of the paramagnetic metal ion ($T_{1\text{obs.}}^{-1}$, $T_{2\text{obs.}}^{-1}$). The paramagnetic effect then is:

$$T_{1p}^{-1} = T_{1\text{obs.}}^{-1} - T_{10}^{-1} \quad (2)$$

$$T_{2p}^{-1} = T_{2\text{obs.}}^{-1} - T_{20}^{-1} \quad (3)$$

$$= \pi(\nu_{\text{fobs.}} - \nu_{\text{fobs.}})$$

$\nu_{\text{fobs.}}$ and ν_{f0} are the linewidth in hertz at half-height with and without Cu(II) respectively.

Because Cu(II) has a long electron-spin relaxation time ($\tau_s^{-1} \sim 10^8 \text{ s}^{-1}$), its dominant effect is line broadening; the relaxation in the bound sphere is so efficient that $1/T_{2M}^2 \gg \Delta\omega_M$, and one can assume that $\Delta\omega_M$, the shift between the resonances of free and bound ligand, is ineffective. For this case, and in the absence of significant outer sphere effects, Swift & Connick (1962) and Luz & Meiboom (1964) have shown that:

$$T_{1p}^{-1} = pq/(T_{1M} + \tau_M) \quad (4)$$

and

$$T_{2p}^{-1} = pq/(T_{2M} + \tau_M) \quad (5)$$

where p is the ratio of metal ions to ligand molecules, q the number of co-ordinated ligands per metal ion, τ_M the average residence time of the ligands in the

bound form, and T_{1M} and T_{2M} are the longitudinal and the transverse relaxation time of the bound ligand respectively. T_{1M} and T_{2M} are given by the classical Solomon-Bloembergen equations (Solomon, 1955; Bloembergen, 1957; Bloembergen & Morgan, 1961).

For the case of slow exchange, $\tau_M \gg T_{1M}, T_{2M}$, the Swift-Connick equation reduces to:

$$T_{1,2p}^{-1} = pq \cdot \tau_M^{-1} \quad (6)$$

and when the exchange is very fast the conditions $\tau_M \ll T_{1M}, T_{2M}$ exist and the limiting equation is:

$$T_{1,2p}^{-1} = pq \cdot T_{1,2M}^{-1} \quad (7)$$

Frequency- and temperature-dependence studies of linewidth provide experimental tests to show if slow or fast exchange is operating. With Cu(II), fast exchange is often assumed (Laussac & Sarkar, 1980a). In this condition, $T_{1p}/T_{2p} = T_{1M}/T_{2M}$ and the evaluation of this ratio is a diagnostic test for the magnetic dipolar mechanism. Indeed, in Cu(II) complexes, T_{1M}^{-1} has been shown to be wholly dipolar and proportional to the inverse sixth power of the distance from the carbon to the metal, whereas T_{2M}^{-1} is not (Espersen & Martin, 1976). If, however, the dipolar term dominates the line-broadening and T_{2M}^{-1} , the ratio $T_{1M}/T_{2M} = 7/6$. If the scalar mechanism is important, $T_{1M}/T_{2M} > 7/6$. In such a case, selective broadening experiments may not reflect the explicit r^{-6} -distance-dependence of the dipolar term, even though they may still be used to determine the sites of Cu(II) binding.

E.p.r. spectra

X-band e.p.r. spectra were obtained on a Bruker ER 200 D spectrometer at 110 and 293 K. Samples were frozen in liquid N₂ in quartz tubes. The variation of the spectrum of Cu(II)-Gly-His-Lys (10:1) as a function of pH was done by titrating the complex at room temperature.

Results and discussion

pH-dependence of chemical shifts

As a preliminary step to studying the bonding properties of Gly-His-Lys, we have monitored the pH-dependence behaviour of the peptide in 2H₂O. Assignments of the chemical shifts to specific protons and carbon atoms were made by combined use of decoupling experiments and titration effects.

Classical pH titration curves of all the proton and carbon resonances are obtained. With protons, the major changes occur in the histidine residue as a result of the charge change in the imidazole ring. Thus C₍₂₎-H and C₍₅₎-H protons titrate at a pK_a well within the range expected for the histidine imidazole ring [numbering in accordance with IUPAC-IUB Commission on Biochemical Nomenclature (1975) Recommendations]. The assign-

ments are in agreement with reports in the literature (Rabenstein *et al.*, 1977).

Carbon resonances show also some important alterations. On increasing pH, the lowest-field carbonyl signal at about 177 p.p.m. shows a downfield chemical-shift change. The pK_a value of this change is 2.6, where the deprotonation of a lysine carboxy group occurs. A rapid downfield chemical shift of the signal near 169 p.p.m. on going from pH 6 to 9 (pK_a 7.9) indicates the deprotonation of the α -amino group, and therefore this resonance was assigned to glycine CO. The histidine C₍₂₎ and C₍₄₎ carbons titrate downfield at pH < 5 and yield 'normal' titration curves (Laussac & Sarkar, 1980b; Iyer *et al.*, 1981). The spectral behaviour of the α - and β -carbon resonances are quite similar to their carbonyl and aromatic carbon resonances, and thus assignments were made by comparison with these resonances. The variation of the pH up to 9.5 reflects deprotonation of the lysine ammonium group (pK_a ~ 10.8 by following lysine C_(δ) chemical shifts).

Studies of spin-lattice relaxation times

The ¹³C T_1 values were determined at 22.63 MHz and 62.86 MHz respectively for the proton-bearing carbon resonances. The nuclear Overhauser enhancement effects performed at 22.63 MHz indicate that only dipole-dipole interactions contribute to ¹³C-H relaxation. Furthermore, all the N T_1 values for the carbons of peptide backbone and for those of the lateral chains are in the range expected for a molecule of this size (Laussac & Sarkar, 1980b).

T_1 values are longer at 62.86 MHz than at 22.63 MHz owing to the expected frequency-dependence of T_1 (Doddrell *et al.*, 1972). The derived correlation time associated with molecular motion in solution was determined from field-dependent studies of T_1 values of protonated carbon atoms. The average ratio of the T_1 value at both frequencies is approx. 1.6, which corresponds to a rotational correlation time of approx. 2.10⁻¹⁰ s, which is the order of magnitude expected for a particle of the size of Gly-His-Lys (Laussac & Sarkar, 1980b).

Cu(II)-glycyl-L-histidyl-L-lysine complexes

Addition of Cu(II) ions to solutions of Gly-His-Lys, in peptide/metal ratios of 500:1, produces significant changes in the ¹³C-n.m.r. spectrum. Fig. 1 shows the effect of Cu(II) at several different pH values. The main interesting observation in this Figure is that each one of the lysine CO₂⁻, glycine CO, histidine C₍₂₎, C₍₄₎ and C₍₅₎, lysine C_(α), glycine CH₂, lysine C_(ϵ), histidine C_(β) and lysine C_(δ) signals have a characteristic linewidth, depending on the specific pH value. The C-terminal end of the peptide is first affected by Cu(II) at pH 2.3; the lysine α -carbon atom shows similar line-broadening com-

Fig. 1. Proton-decoupled Fourier-transform ^{13}C -n.m.r. spectra of $^2\text{H}_2\text{O}$ solutions containing 0.23 M-Gly-His-Lys and 0.46 mM-Cu(II)

The pH of the solutions was (a) 2.3, (b) 5.9 or (c) 10.4.

pared with lysine CO_2^- . This is the expected result if one considers that in acidic solution the carboxy group is capable of interacting with a Cu(II) ion. The fraction of carboxylic acid that is protonated is in rapid equilibrium with the deprotonated one, accounting for the overall broadening. As the pH is raised (5.9), the imidazole $\text{C}_{(2)}$, $\text{C}_{(4)}$ and $\text{C}_{(5)}$ and histidine $\text{C}_{(\beta)}$ signals exhibit remarkable line-broadening at pH close to the pK_a value of the N-3 imidazole nitrogen atom. It is also noted in Fig. 1 that both the *N*- and the *C*-terminal ends of the Gly-His-Lys molecule (glycine CO, glycine CH_2 and lysine CO_2^-) are affected by metal at this same pH. With increasing pH, both lysine CO_2^- and lysine $\text{C}_{(\alpha)}$ signals again sharpen when the pH rises above 7.5. At still higher pH values (10.4), imidazole $\text{C}_{(2)}$, $\text{C}_{(4)}$ and $\text{C}_{(5)}$, histidine $\text{C}_{(\beta)}$, glycine CO and glycine $\text{C}_{(\alpha)}$ remain largely broadened, but the more interesting observation is that lysine $\text{C}_{(\delta)}$ and lysine

$\text{C}_{(\epsilon)}$ signals are very much affected by Cu(II) ions, as the ϵ -amino group becomes deprotonated. Results in basic solution are very different from the observation encountered with the albumin *N*-terminal tripeptide: Asp-Ala-His-NHMe (Laussac & Sarkar, 1980a). Indeed, no line broadening was observed for any carbon resonances above pH 10. The sharpening of the signals with increasing pH was explained by the slowing down of the exchange rate (with respect to the n.m.r. time scale), which renders the metal ion ineffective in interacting with excess ligand, so that all the resonances are observed (Laussac & Sarkar, 1980a).

Overall results are supported by the data obtained from ^1H -n.m.r. experiments. The effect of pH titration for the Cu(II)-Gly-His-Lys system is exactly what one should expect.

In order to elucidate the mechanism of the relaxation time, $T_{2\rho}$, at different pH values, we studied

the temperature- and frequency-dependence of line-broadening. Indeed, the effect of temperature on the observed values of T_{2p}^{-1} can be used to determine the dominant (slowest) process (Luz & Meiboom, 1964). The logarithm of the broadening of the $C_{(2)}\text{-H}$, $C_{(5)}\text{-H}$ and glycine CH_2 resonances by Cu(II) as a function of the inverse of the temperature is shown in Fig. 2 at pH 6.80. The following features are noticeable in the curves. (i) For the $C_{(2)}\text{-H}$ and $C_{(5)}\text{-H}$ protons, as the temperature is raised, T_{2p}^{-1} passes through a maximum at approx. 38°C and then decreases with further increase in temperature. (ii) A plot of $\log T_{2p}^{-1}$ versus $10^3/T$ for glycine CH_2 resulted in a straight line over the temperature region measured. These results show that at a low temperature ($20\text{--}38^\circ\text{C}$) $C_{(2)}\text{-H}$ and $C_{(5)}\text{-H}$ data points fall into the exchange region given by eqn. (6) whereas the other data points fall into the fast-exchange region (eqn. 7). On the other hand, glycine CH_2 protons fall into the slow-exchange region over the entire temperature range.

In basic solution (pH 10.0), the temperature-dependences of T_{2p} for all these protons are very similar to that observed near physiological pH. Furthermore, the lysine $C_{(\epsilon)}$ protons seem to be unaffected. In the same way, the ^{13}C values of the line-broadening were measured at two different field strengths. At pH 5.30 and 10.10, the ratio T_{2p}^{-1} (22.6 MHz)/ T_{2p}^{-1} (62.86 MHz) shows a very small frequency-dependence (Table 1). These data are far from the predicted square of the ratio of two frequencies, i.e. $(62.86/22.6)^2 = 7.7$ (Dwek, 1973). Thus T_{2p}^{-1} values for the $\text{Cu(II)}\text{-Gly-His-Lys}$ system are independent of observation frequency, and no shift in the resonances of the free ligand was observed even at the highest Cu(II) concentrations employed. The lack of any frequency-dependence on

Fig. 2. Temperature variation of experimental (T_{2p}) relaxation time for $\text{Cu(II)}\text{-Gly-His-Lys}$ at pH 6.80. The concentration of Gly-His-Lys was 0.23 M and that of Cu(II) was 0.46 mM. Δ , Gly CH_2 ; $\circ$, $C_{(2)}\text{-H}$; $\square$, $C_{(5)}\text{-H}$.

the linewidth and the absence of chemical shift indicate that we are outside the intermediate-exchange region (Dwek, 1973).

In experiments with Cu(II) , the ligand is always in very large excess. The average stoichiometry of the species present is unknown, and the interpretation assumes only that exchange of free ligand and bound ligand is fast on the n.m.r. time scale, i.e. each resonance corresponds to the weighted average of unbound and bound ligand. [Recently we have observed an unusual behaviour of a copper-bound peptide. Indeed, in the case of Cu(II) -binding to the 24-residue peptide fragment from the *N*-terminal of human serum albumin, it is necessary to use a very low ligand/ Cu(II) ratio (approx. 2:1) to observe the broadening. This unusual effect strongly suggests that the system is in slow exchange (J.-P. Laussac & B. Sarkar, unpublished work).]

Results obtained from temperature-variation experiments are opposite to that expected in a fast-exchange limit. This may be due to the fact that in the observed pH region the Cu(II) ions compete with different species. Hence this can be explained easily by the presence of major and minor species, as

Table 1. T_{2p}^{-1} (22.63 MHz)/ T_{2p}^{-1} (62.86 MHz) values for $\text{Cu(II)}\text{-Gly-His-Lys}$ complex at two different pH values

The peptide/ Cu(II) molar ratio was 500:1.

Resonance	T_{2p}^{-1} (22.63 MHz)/ T_{2p}^{-1} (62.86 MHz)	
	pH 5.30	pH 11.10
Gly $C_{(\alpha)}$	1.0	0.8
Gly CO	1.1	0.6
His $C_{(\beta)}$	1.2	1.5
His $C_{(2)}$	0.8	1.8
His $C_{(4)}$	0.7	1.3
His $C_{(5)}$	0.6	1.3
Lys $C_{(\alpha)}$	1.1	1.1
Lys $C_{(\delta)}$	1.0	1.3
Lys $C_{(\epsilon)}$	1.0	1.2
Lys CO_2^-	2.2	1.2

Table 2. ^{13}C spin-lattice and transverse relaxation times of Gly-His-Lys in the presence of Cu(II) at 22.63 MHz. The peptide/ Cu(II) molar ratio was 500:1; the pH was 7.4.

Resonance	T_{1p}^{-1} (s^{-1})	T_{2p}^{-1} (s^{-1})	T_{1p}/T_{2p}
Gly $C_{(\alpha)}$	0.28	60	210
His $C_{(\alpha)}$	0.39	25	65
His $C_{(\beta)}$	0.31	60	190
His $C_{(2)}$	0.33	430	1300
His $C_{(5)}$	0.36	390	1100
Lys $C_{(\alpha)}$	0.30	55	180
Lys $C_{(\epsilon)}$	0.42	20	50

suggested by several workers (Kuroda & Aiba, 1979; Gairin *et al.*, 1982). This is consistent with our earlier observation of multiple species in the Cu(II)–Gly–His–Lys system (Lau & Sarkar, 1981). It is well established that Gly–His–Lys forms a very stable complex with Cu(II). Since the metal is tightly bound to the ligand, very slow dissociation occurs, and the observed n.m.r. spectrum represents the excess unbound Gly–His–Lys, which is not affected by the inert Cu(II)–Gly–His–Lys complex. In this condition, line-broadening is not due to the stable major complex, which may have a large τ_M , but is ascribed to minor species with fast ligand-exchange rate. In this case, selective broadening studies with Cu(II) should be undertaken with caution and are not generally useful in elucidating binding sites of the major species.

Values of the ^{13}C longitudinal and transverse relaxation rates obtained at 22.63 MHz are tabulated as $T_{1\rho}^{-1}$ and $T_{2\rho}^{-1}$ in Table 2. The very high $T_{1\rho}/T_{2\rho}$ ratio and the lack of any frequency-dependence of the linewidths indicate that the scalar term predominates in the equations for $T_{2\rho}^{-1}$ for all the carbon atoms that are affected by Cu(II) and that the r^{-6} -dependent dipolar term is not the predominant cause of relaxation. Thus the selectivity in signal broadening can be used qualitatively to locate the binding site of a paramagnetic Cu(II) ion, but this method is unlikely to provide quantitative structural information (Espersen & Martin, 1976).

E.p.r. experiments

E.p.r. studies of Cu(II) complexes provide very useful information about the symmetry and the bonding of the central metal ion with the surrounding ligands. Fig. 3 shows typical e.p.r. spectra of aqueous Cu(II) solutions containing a 10-fold excess of ligand at different pH values and presents clear evidence of formation of paramagnetic complexes. [Similar results are obtained with a Gly–His–Lys/Cu(II) ratio of 100:1.] Under acidic conditions, a broad absorption results from the rapid tumbling of the relatively small hydrated Cu(II) ions. Co-ordination of lysine CO_2^- is not expected to change much the broad absorption line. Above pH 3, when a stronger chelate-like complex begins to form, additional absorptions appear at higher fields and we observe the partly resolved four-line pattern of a typical Cu(II)–peptide complex (Fig. 3). Over a wide pH range, the observed spectrum is almost entirely due to similarly co-ordinating species with $g_0 = 2.121$ at pH 4.1 and 2.110 at pH 10.7 and an isotropic hyperfine coupling constant averaging 8.5 mT in the pH interval 4.1–10.7. Starting at pH 5.5, the narrowest absorption near 340 mT shows superhyperfine structure, which comes out sharply in the spectra in the pH range 6.0–9.6. Seven superhyperfine lines could clearly be discerned that are

Fig. 3. E.p.r. spectra of solutions containing Gly–His–Lys and Cu(II) (ligand/metal ratio 10:1) at 293 K at different pH values

The pH was (a) 3.2, (b) 4.2, (c) 7.0, (d) 8.7 or (e) 10.7.

due to the three interacting ^{14}N nuclei co-ordinating strongly to Cu(II) (Wiersema & Windle, 1964). The g -values and Cu(II) hyperfine splittings agree well with a slight increase in metal–ligand bond strength as the pH increases. Under strongly basic conditions, the superhyperfine structure is lost. This loss of resolution, on the basis of correlating visible-spectral evidence, can be attributed, for example, to the involvement of the fourth nitrogen nuclei around Cu(II).

Frozen solutions (120 K) of the Cu(II)–Gly–His–Lys complexes give resolved anisotropic e.p.r. spectra. In liquid solution the complexes are randomly oriented and rotating freely, whereas in the frozen state they are fixed. From this, the values of $g_{\parallel}$ and $A_{\parallel}$ can be measured accurately. The nuclear hyperfine structure $A_{\parallel}$ is resolved on the $g_{\parallel}$ part of

the spectrum. However, superhyperfine structure of the $g_{\perp}$ position is poorly resolved. Nevertheless, the e.p.r. parameters in medium pH, $A_{\parallel} = 19.5$ mT and $g_{\parallel} = 2.21$, fit well with previously determined data on co-ordination spheres including one oxygen atom and three nitrogen atoms (Formicka-Kozłowska *et al.*, 1977). Peisach & Blumberg (1974) have pointed out that $A_{\parallel}$ and $g_{\parallel}$ vary with the composition of the ligand atoms bound to the Cu(II). Thus a structural assignment of a given Cu(II) complex can be made on the basis of its position in the plot of $A_{\parallel}$ versus $g_{\parallel}$, e.g. the effect of increased electron-donating ability on the e.p.r. parameters is to decrease $g_{\parallel}$ and increase $A_{\parallel}$. Our results are compatible with three-nitrogen and one-oxygen (3N, 1O) ligation around Cu(II). The e.p.r. spectrum is typical of axial symmetry and can be assigned to that of a mononuclear Cu(II) complex with $d_{x^2-y^2}$ ground state.

The relatively large $A_{\parallel}$ value and the observations of the nitrogen superhyperfine splitting ($A_N = 1.1$ mT) confirm that the ligand field is likely to be square-planar around the Cu(II) atom.

In conclusion, if our e.p.r. results clearly show that the co-ordination around Cu(II) in the major species is square-planar involving the carboxy oxygen atom and three nitrogen atoms, our n.m.r. data should be interpreted with caution. Indeed, broadening reflects only the presence of very rapidly exchanging minor species and the results are not useful in elucidating binding sites of stable major species, since the broadening would not be observable at the high ligand/metal ion ratios employed.

Note added in proof (Received 1 December 1982)

After the present paper was submitted for publication, a paper on the 'Structure of glycyl-L-histidyl-L-lysine-copper(II) in solution' was published by Freedman *et al.* (1982). These authors reported that Cu(II)-Gly-His-Lys forms a 1:1 complex at physiological pH. Their conclusion is based on e.p.r. experiments performed in ethylene glycol/water (1:1, v/v), which is suspect for the exact determination of pH. According to our earlier studies of the Cu(II)-Gly-His-Lys system in aqueous solution, we have shown 100% of 1:1 complex at approx. pH 5, but at physiological pH there is about 60% of 1:2 (bis species) complexes (Lau & Sarkar, 1981). A small variation in pH, due to the use of the ethylene glycol/water system near neutrality, can produce large changes in complex species, raising the possibility of serious errors in the estimation of the stoichiometry. Furthermore, if their e.p.r. determination of the stoichiometry by titration is correct, the error is at least $\pm 10\%$, since it is difficult to measure the amplitude of $g_{\perp}$ because of the very broad peaks.

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