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Synthesis and copper(II)-binding properties of the *N*-terminal peptide of human α-fetoprotein

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The *N*-terminal native sequence tripeptide of α-fetoprotein, L-threonyl-L-leucyl-L-histidine *N*-methylamide, was synthesized and its interaction with Cu(II) ions was investigated by potentiometric titration at 25 °C in 0.15 M-NaCl and by visible-absorption, e.p.r. and n.m.r. spectroscopy. Analyses of the results in the pH range 4–10 indicated the presence of multiple complex species in solution: MHL, $MH_{-2}L$, MHL_2, ML_2 and $MH_{-1}L_2$, where M, H and L represent metal ion, proton and ligand anion respectively. Only the species $MH_{-2}L$ and $MH_{-1}L_2$ are present in significant amounts at physiological pH. The results of the visible-absorption spectroscopy are consistent with the findings of species distribution that $MH_{-2}L$ is the major complex species detected above physiological pH that has the spectral characteristics of $\lambda_{max.} = 523$ nm and $\epsilon_{max.} = 98\ M^{-1} \cdot cm^{-1}$. The nine superhyperfine lines in e.p.r. spectra of the major species $MH_{-2}L$ strongly support the co-ordination of four nitrogen atoms by Cu(II). Both ^1H- and ^{13}C-n.m.r. studies suggest that the species $MH_{-2}L$ is a square-planar complex. The results from the equilibrium-dialysis experiments showed that this peptide is able to compete with albumin for Cu(II) ions. At equimolar concentrations of albumin and the peptide, about 52% of the Cu(II) was bound to the peptide. The possibility that α-fetoprotein plays an important role as the Cu(II)-transport protein in fetal life is discussed.

INTRODUCTION

α-Fetoprotein (AFP) is a major serum protein synthesized during fetal life [1,2] and its content decreases rapidly during early postnatal life. Trace amounts of AFP are present in normal adult serum, ranging from 2 to 25 ng/ml [3,4]. Any increase in serum concentration signals pathological conditions [5,6]. Although the exact function of AFP is unknown, its physicochemical properties, including storage and transport of certain small ligands, are similar to those of serum albumin [7–10]. There is also approx. 40% of sequence homology between these two proteins. However, human serum albumin (HSA) concentrations follow an opposite pattern, increasing from low values early in fetal development to high, approximately constant, values after birth and in adult life [5,11]. The combined observations have led to the suggestion that AFP possibly serves as a fetal equivalent of HSA.

Earlier studies have indicated that human AFP can bind Cu(II) and Ni(II) ions and that AFP may also function as a carrier protein for Cu(II) and Ni(II) ions in fetal life [12]; but any detailed study of the binding site is lacking. The site of metal-ion binding probably occurs at the *N*-terminal segment of the protein. However, the *N*-terminus of human AFP is composed of two major components, namely types 1 and 2 with the sequences Ser-Thr-Leu-His-Arg- and Thr-Leu-His-Arg- respectively [13]. According to our earlier studies, the histidine residue at the third position from the *N*-terminus in the type 2 sequence should provide similar Cu(II)-ion-binding properties to those observed for HSA [14]. In the present paper the synthesis of the tripeptide Thr-Leu-His-$NHCH_3$, which is a major tripeptide sequence of native AFP with the *C*-terminus protected to resemble the protein more closely, and its detailed Cu(II)-ion-binding studies are reported and discussed in comparison with the synthetic tripeptide models for HSA.

EXPERIMENTAL

Materials

Crystalline human serum albumin obtained from Hoechst Pharmaceutical Co. (c/o Calbiochem–Behring Corp., La Jolla, CA, U.S.A.) was used without further purification. $^{67}CuCl_2$ (specific radioactivity 2.25 Ci/mg) was purchased from Los Alamos National Laboratory (Los Alamos, NM, U.S.A.). Dialysis membrane was from Union Carbide (Toronto, Ont., Canada). Potassium hydrogen phthalate was obtained from the National Bureau of Standards (Washington, DC, U.S.A.). Z-L-Leu-ONSu,HCl and Boc-L-Thr-ONSu were from Bachem A.G. (Bubendorf, Switzerland). Molecular sieve type 4A was from BDH Chemicals (Toronto, Ont., Canada). $Cu(ClO_4)_2,6H_2O$ was purchased from Ventron (Karlsruhe, West Germany). Sodium 4,4-dimethyl-4-silapentane-1-sulphonate was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). ^2H_2O (99.93%) was obtained from the Commissariat à l'Energie Atomique (Gif-sur-Yvette, France). Precoated thin-layer sheets (Polygram Cel 300 and Sil G) were from

Abbreviations used: AFP, α-fetoprotein; HSA, human serum albumin; DAH, L-aspartyl-L-alanyl-L-histidine *N*-methylamide, the *N*-terminal native sequence tripeptide of human serum albumin; GGH, glycylglycyl-L-histidine; TLH, L-threonyl-L-leucyl-L-histidine *N*-methylamide, the *N*-terminal native sequence tripeptide of α-fetoprotein. Z, benzyloxycarbonyl; Boc, t-butoxycarbonyl; ONSu, succinimido-oxy.

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Brinkmann Instruments (Rexdale, Ont., Canada). The solvent systems used were as follows: solvent A, chloroform/methanol/acetic acid (17:2:1, by vol.); solvent B, butan-1-ol/acetone/water/acetic acid (4:3:2:1, by vol.); solvent C, butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.); solvent D, propan-2-ol/water (7:3, v/v). Melting points are reported uncorrected. 1,2-Dimethoxyethane was dried over a molecular sieve overnight and used fresh. Triethylamine was purified as previously described [15]. All other reagents were of analytical grade.

Synthesis of L-threonyl-L-leucyl-L-histidine *N*-methylamide (TLH)

To a cooled solution of L-His-OMe,HCl (4.78 g, 23.24 mmol) in anhydrous 1,2-dimethoxyethane (100 ml) were added triethylamine (3.3 ml, 23.55 mmol) first and then Z-L-Leu-ONSu (8.02 g, 22.16 mmol). The mixture was stirred at room temperature overnight. The residue, after removal of solvent, was diluted with water and extracted with four 75 ml portions of ethyl acetate. The combined extracts, after usual work-up procedures, were dried to give a white solid of Z-L-Leu-L-His-OMe (5.54 g; 60% yield; m.p. 145–148 °C). It was homogeneous on t.l.c. (Sil G) in solvent systems A and B.

A solution of Z-L-Leu-L-His-OMe (4.0 g) in 150 ml of methanol cooled in an ice bath was saturated with monomethylamine gas (approx. 6 h). T.l.c. (Sil G) in solvent A showed the reaction to be complete. The product, Z-L-Leu-L-His-NHMe, was recrystallized from 95% ethanol (3.42 g; 85% yield). This was dissolved in methanol (75 ml) and acetic acid (5 ml) and hydrogenated for 5 h in the presence of 10% Pd/C (300 mg). The reaction product, L-Leu-L-His-NHMe,2CH_3CO_2H was passed through a silica-gel column (2.5 cm × 100 cm) in solvent B. The fractions containing the dipeptide were pooled and the solvent was removed to yield a white solid (2.14 g; 65% yield).

The tripeptide Boc-L-Thr-L-Leu-L-His-NHMe was prepared by coupling Boc-L-Thr-ONSu (1.23 g, 3.88 nmol) and the dipeptide (1.48 g, 3.68 mmol) via the active ester method again. The crude product was purified through the silica-gel column in solvent B, and then treated with anhydrous DME (25 ml) and acetic acid (3 ml) and cooled in an ice bath, and then anhydrous HCl (5.7 M) in 1,2-dimethoxyethane (15 ml) was added. Anhydrous diethyl ether (200 ml) was then added, and the precipitated solid was filtered, washed with diethyl ether three times and dried *in vacuo* over KOH (0.75 g; 45% yield). This solid was chromatographed on a cellulose column (2.5 cm × 100 cm) in solvent D. Fractions containing the pure tripeptide were pooled and dried (0.62 g; m.p. 160–164 °C). This compound showed a single spot on t.l.c. in solvents C (Sil G) and D (Cel 300), the correct n.m.r. spectrum (in ^2H_2O) and amino acid analysis (Thr/Leu/His: 0.92:1:0.94). Only very trace impurity was found in this sample by h.p.l.c. on a C_{18} μBondapak column in 10% (v/v) methanol.

Potentiometric titration

The titrations were performed on a Radiometer automatic titration assembly thermostatically maintained at 25 °C. NaOH, prepared carbonate-free and kept under Ar atmosphere, was standardized against primary acid potassium hydrogen phthalate. $CuCl_2$ was dissolved in 1 mM-HCl and standardized complexometrically against EDTA with murexide as indicator. The peptide stock solution was prepared in water and stored at 4 °C when not in use. All the solutions contained 0.15 M-NaCl and known amounts of HCl to lower the starting pH below that of metal-ion binding and were then titrated with 0.100 M-NaOH from pH 2.7 to 10.5. In the free peptide system, the concentrations varied between 0.2276 and 0.9104 mM. In the Cu(II)–peptide system, the peptide concentrations varied between 0.4092 and 0.8184 mM and the metal ion concentrations varied between 0.114 and 0.342 mM. The molar ratio of peptide to Cu(II) ions ranged from 1.79:1 to 5.38:1 in the metal ion variation and from 2.33:1 to 3.59:1 in the ligand variation. All the calculations were carried out by a sequential use of programs on a microcomputer [16].

Equilibrium dialysis

In the absence of human AFP, HSA was used instead in the competition for Cu(II) ions between the tripeptide and the protein. This was performed in 0.1 M-*N*-ethylmorpholine/HCl buffer, pH 7.63 and I 0.16, at 6 °C by the equilibrium-dialysis technique [17]. One half-cell contained the stock solution of Cu(II)–albumin (0.145 mM) mixed with $^{67}CuCl_2$, and the other half-cell contained various amounts of the peptide. The molar ratio of the peptide to Cu(II)–albumin ranged from 0.1:1 to 2.6:1. ^{67}Cu radioactivity was measured before and after dialysis.

Visible-absorption spectra

The visible-absorption spectra of the Cu(II)-ion complexes were recorded on a Beckman Acta model MVI spectrophotometer as a function of pH in 0.15 M-NaCl at 23 °C. The Cu(II) peptide molar ratio was kept at 0.84:1.

E.p.r. spectra

X-band spectra were obtained on a Bruker ER 200D spectrometer at 110 and 293 K. Samples (10 mM) were frozen in liquid N_2 in quartz tubes. The variation of the spectrum of Cu(II)–tripeptide as a function of pH was carried out by titrating the complex at room temperature.

^1H- and ^{13}C-n.m.r. spectra

^1H-n.m.r. spectra at 250 MHz and ^{13}C-n.m.r. spectra at 62.8 MHz were recorded on a Bruker WM250 spectrometer equipped with an Aspect 3000 computer system. The usual spectrometer conditions were 2800 Hz sweep width, 1s cycle time, 16 k data points and 250 scans for ^1H and 1500 Hz sweep width, 0.5 s relaxation delay, 20 μs (60 °) pulse length, 16 k data points and 40000–70000 scans per sample (5 mM) for ^{13}C. Both ^1H and ^{13}C chemical shifts were measured (in p.p.m.) with sodium 4,4-dimethyl-4-silapentane-1-sulphonate for external reference calibration. The signal for the external reference was then recorded in the computer of the spectrometer.

RESULTS

Determination of species distribution and stability constants

The general equilibrium involving metal ion M, proton H and ligand anion L can be represented as:

$$pM + qH + rL \rightleftharpoons M_pH_qL_r \quad (1)$$

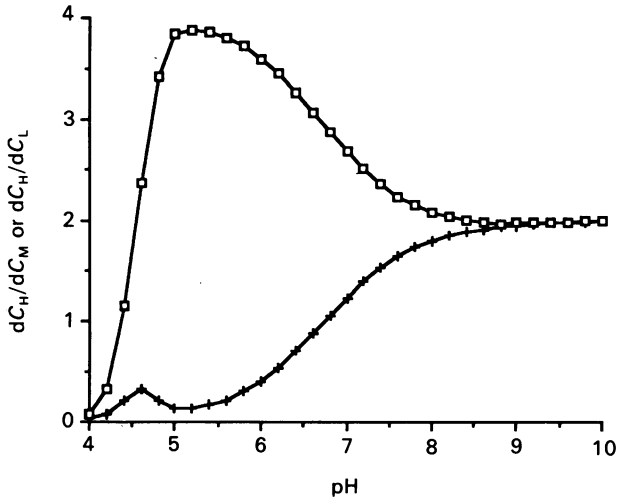

Fig. 1. Proton liberation as a function of pH for the system Cu(II)–TLH

□, dC_H/dC_M; +, dC_H/dC_L.

where p, q and r are the stoichiometric quantities of M, H and L respectively. The stabilities of the species formed are expressed as the equilibrium constants β_{pqr} in terms of concentrations at constant ionic strength, temperature and pressure:

$$\beta_{pqr} = \frac{[M_pH_qL_r]}{m^p \cdot h^q \cdot l^r} \quad [2]$$

where m, h and l are the concentrations of free metal ion, hydrogen ion and anionic ligand respectively. The titration results were obtained with solutions containing different concentrations of metal ion (C_M), hydrogen ion (C_H) and anionic ligand (C_L). The mathematical analyses of the titration data were carried out by a series of computer programs, the detailed description of which has been published previously from our laboratory [16].

Complexing of Cu(II) ions by this tripeptide begins slightly below pH 4 and is complete around pH 5.2. The proton liberation profiles are shown in Fig. 1. For the species selection of the complexes formed, the following values for p, q and r were considered: $p = 1$; $q = 2, 1, 0, -1, -2$; $r = 1, 2$. The calculations indicated that combination of the complex species MHL, $MH_{-2}L$, MHL_2, ML_2 and $MH_{-1}L_2$ gave the best overall mathematical fit. Their stability constants expressed as $\log\beta_{pqr}$ are listed and compared with those of DAH and GGH in Table 1, together with the pK_a values of TLH. The species distribution as a function of pH is shown in Fig. 2.

Visible-absorption spectra

The visible-absorption spectra of a solution containing 8.76 mM-Cu(II) ions and 10.4 mM-TLH in 0.15 M-NaCl were recorded at various pH values. The results indicate that the absorption energies do not seem to change much; however, the absorbances increase as the pH values rise. Above pH 8.82 no significant changes are observed. This is consistent with the result of species distribution that $MH_{-2}L$ is the only complex species detected above pH 9. It has the spectral characteristics of $\lambda_{max.} = 523$ nm and $\epsilon_{max.} = 98\ M^{-1} \cdot cm^{-1}$.

Table 1. pK_a and log (stability constants) [$\log(\beta_{pqr})$] of the complex species $M_pH_qL_r$ for the Cu(II)–TLH system in 0.15 M-NaCl at 25 °C and their comparison with those of the Cu(II)–GGH and Cu(II)–DAH systems

				$\log\beta_{pqr}$		
p	q	r	pK_a	TLH	GGH [18]	DAH [15]
0	2	1	6.24±0.02			
0	1	1	7.44±0.03			
1	1	1		11.17		
1	0	1			7.55	8.39
1	−1	1			2.68	
1	−2	1		−1.34	−1.92	−0.55
1	2	2			25.81	
1	1	2		19.18	20.64	
1	0	2		15.55	16.68	
1	−1	2		9.60	9.73	10.01
1	−2	2			1.43	

Equilibrium-dialysis studies

The distribution of ^{67}Cu radioactivity at equilibrium indicates that the tripeptide TLH is able to compete with albumin for Cu(II) ions. As shown in Fig. 3, more than 50% of the Cu(II) ions is bound to the peptide at equimolar concentrations. At high peptide/albumin ratios, less than 100% of Cu(II) ions is bound to the peptide. This may imply the formation of a ternary peptide–Cu(II)–albumin complex.

^1H- and ^{13}C-n.m.r. studies

All of the ^1H and ^{13}C signals arising from the atoms of the backbone and on the side chains of TLH have been assigned unambiguously by combined use of decoupling experiments and titration effects.

TLH has two functional groups that are expected to ionize as the pH is raised from 2.50 to 10.95. They

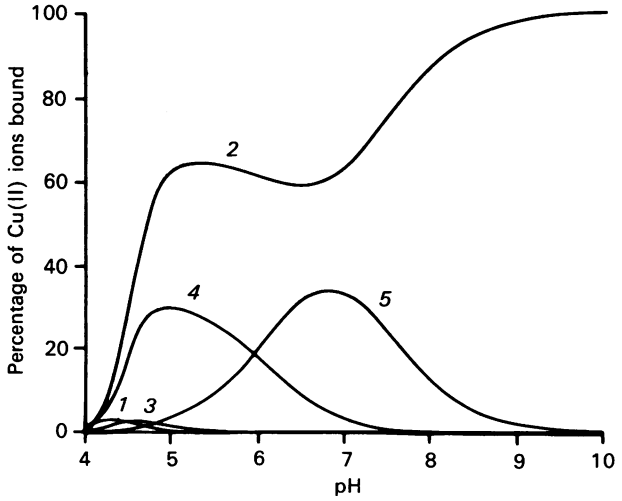

Fig. 2. Species distribution as a function of pH for the system Cu(II)–TLH

Curve *1*, MHL; curve *2*, $MH_{-2}L$; curve *3*, MHL_2; curve *4*, ML_2; curve *5*, $MH_{-1}L_2$.

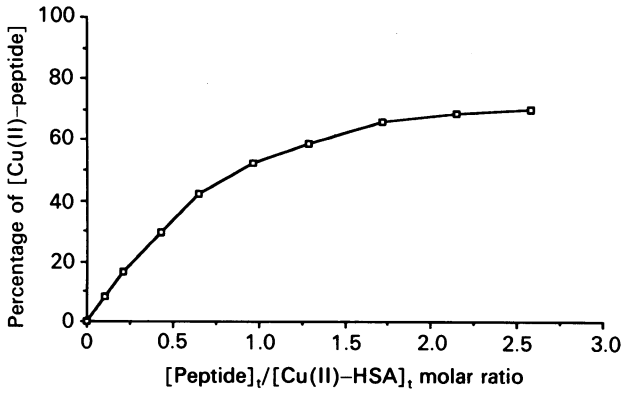

Fig. 3. Competition of TLH with albumin for Cu(II) in 0.1 M-*N*-ethylmorpholine/HCl buffer, pH 7.63 and *I* 0.16 at 6 °C

[Cu(II)–HSA]$_t$ was 0.145 mM.

include the Thr *N*-terminus (pK_a approx. 7.30) and the His imidazole ring (pK_a approx. 6.40).

It is noteworthy that the histidine C-2 and C-4 carbon atoms titrate downfield and yield 'normal' titration curves [19,20]. The large titration effect of the quarternary His C-4 compared with the His C-2 and the unaffected His C-5 suggest a predominance of the N-1–H tautomeric form as proposed by Reynolds *et al.* [21].

Addition of Cu(II) ions to solutions of ligand, in peptide/metal ion ratios of 10:1, produces significant changes in the ^{13}C-n.m.r. spectrum. Fig. 4 shows the effect of Cu(II) ions at several different pH values. The interesting observation is that each of Thr CO, Leu CO, His CO, His C-2, C-5 and C-4, Thr C-α, C-β and C-γ and His C-α and C-β signals has a characteristic linewidth depending on the specific pH value. At pH 4.30 all of these resonances are affected. The Thr CO and His C-2, C-5, C-4 and C-β are broadened beyond detection and disappear in the noise. As the pH is raised to 6.80, the Thr CO, Thr C-α and C-β and His C-β signals exhibit remarkable line-broadening and His C-2, C-5 and C-4 signals are still broadened beyond detection. When the pH was further raised to 10.10 all the resonances were again observed with a linewidth less than 20 Hz.

Overall results are supported by the data obtained from ^{1}H-n.m.r. experiments. Adding Cu(II) ions to a ^2H_2O solution of peptide causes important paramagnetic perturbations as in the ^{13}C-n.m.r. spectra. The induced line-broadening increases together with Cu(II) ion concentration. This effect is pH-dependent. At a TLH/Cu(II) ion ratio as low as 5:1, there are large perturbations in the spectrum at pH 4.75. Noteworthy is the fact that all the three C-α–H signals are similarly affected while the –NH–CH_3 resonance remains sharp. At this pH, the imidazole protons are broadened beyond detection. In neutral solution these signals remain largely broadened or disappear completely in the noise. Finally, in strongly basic conditions, all the signals appear again and sharpen when the pH rises above 10.0. Recently, a similar observation has also been made with a cyclic peptide [22] and a linear peptide [23]. 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 makes the metal ion ineffective in interacting with excess ligand, so that all the resonances are observed.

E.p.r. spectra of Cu(II)–peptide

At room temperature, random motion of solute molecules yields an isotropic spectrum from which g_0 and the nuclear hyperfine structure constant A_0 can be

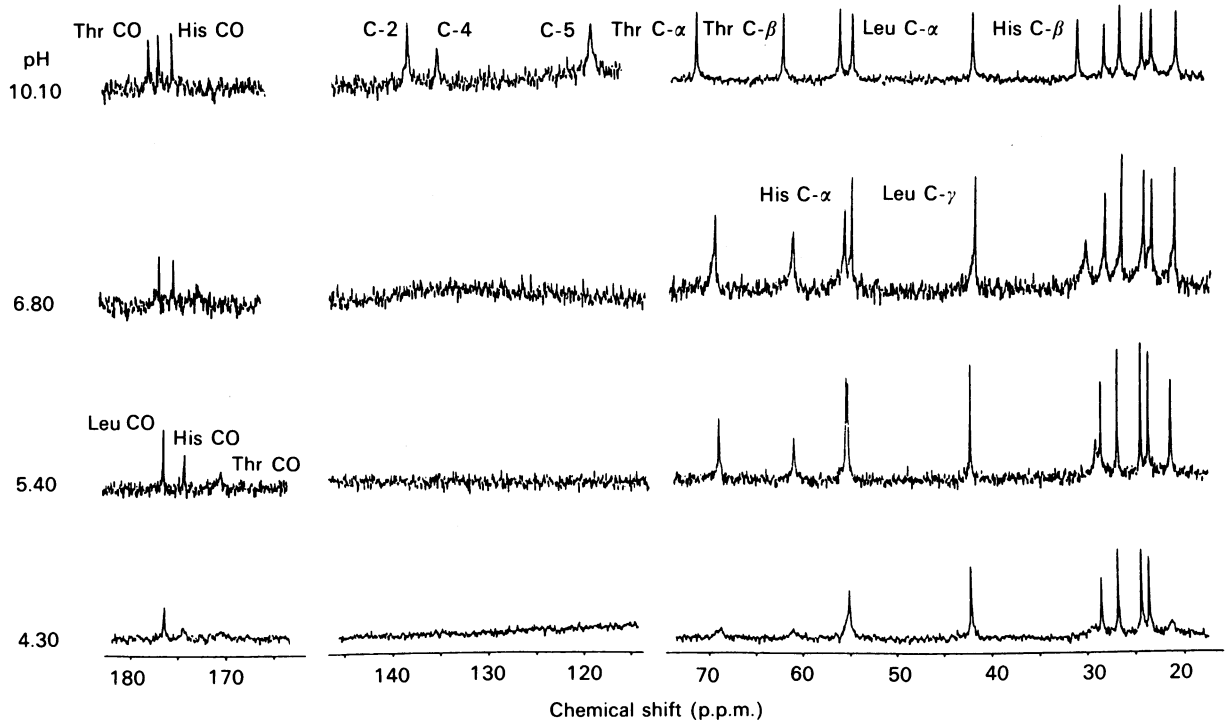

Fig. 4. ^{13}C-n.m.r. spectra (62.8 MHz) of ^2H_2O solutions containing 50 mM-TLH and 5 mM-Cu(II) ions at different pH values

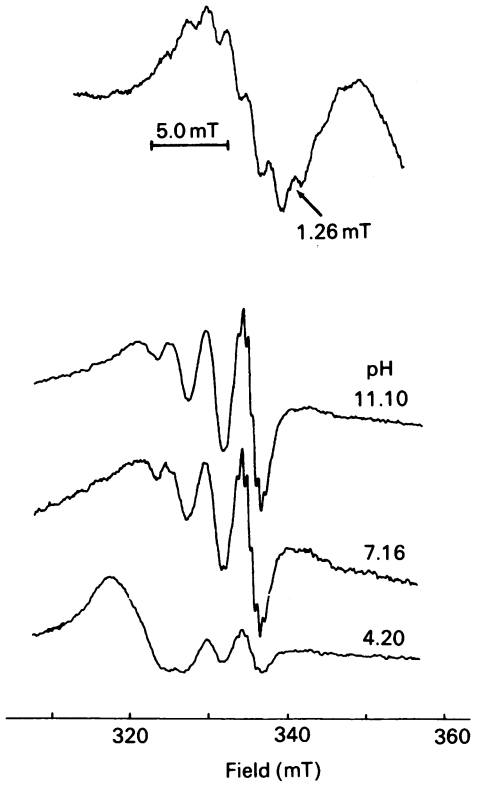

Fig. 5. E.p.r. spectra of 1:1 molar ratio solutions of TLH and Cu(II) ions at different pH values at room temperature

The top spectrum shows a nitrogen nuclear superhyperfine structure on an expanded scale.

measured. Thus at pH 4.20, 7.16 and 11.20 the observed spectra are entirely due to a species with $g_0 = 2.090$ and an isotropic hyperfine coupling constant of about 9.6 mT. Furthermore, Fig. 5 clearly indicates that the e.p.r. splitting corresponds to the superhyperfine interaction with neighbouring nuclei, namely nitrogen (^{14}N, $I = 1$) atoms. Frozen solutions (110 K) of the Cu(II)–TLH complex give resolved anisotropic e.p.r. spectra. In liquid solution the complex is randomly oriented and rotating freely, whereas in the frozen state it is fixed. From this, the values of $g_{\parallel} = 2.177$, $g_{\perp} = 2.046$ and $A_{\parallel}$ 21.5 mT can be measured accurately in the acidic from basic milieu.

DISCUSSION

The tripeptide TLH has two titratable protons in the pH range studied (2.7–10.5). They are attributed to the imidazole and amino groups, with pK_a values of 6.242 and 7.443 respectively. The tripeptide starts to bind Cu(II) ions at a pH slightly below 4, and liberates rapidly to a maximum of about 4 protons in a range of less than 2 pH units and levels off at a value of 2 at high pH. The 4 protons can be attributed to those from the imidazole, amino and the two peptide amide groups, the same as that for the systems DAH and GGH [15,18]. Among the five complex species detected, MHL and MHL_2 are minor (less than 5%) and occurred at low pH, and only the major species $MH_{-2}L$ and two bis-complexes ML_2 and $MH_{-1}L_2$ are present in significant amounts. As $MH_{-2}L$ is the sole complex species found at high pH, the result from the proton-displacement profile suggests that this species would have a similar binding structure to that proposed for the major 1:1 Cu(II) complexes of the tripeptides DAH and GGH, i.e. involving α-amino, imidazole and two peptide nitrogen atoms co-ordinating to the central Cu(II) ion in a square-planar configuration. This contention is consistent with the results obtained from the visible-absorption spectrum, which shows the maximal absorption at 523 nm with the value of $\epsilon_{max.}$ as 98 $M^{-1} \cdot cm^{-1}$ at pH around and above 9. In the pH range studied all the visible-absorption spectra show the maximal absorption around 520–530 nm, indicating the co-ordination of four nitrogen atoms around the square-planar Cu(II). These results indicate that both bis-complexes ML_2 and $MH_{-1}L_2$ have similar spectral characteristics to those of the major species $MH_{-2}L$. In fact, the calculation shows that the complex $MH_{-1}L_2$, the only other significant species present at physiological pH (approx. 25%), has a $\lambda_{max.} \sim 523$ nm and $\epsilon_{max.} \sim 88$ $M^{-1} \cdot cm^{-1}$.

Cu(II)-induced changes in the paramagnetic relaxation of ^1H and ^{13}C can be understood by using the well-known equations of Solomon & Bloembergen [24] and Luz & Meiboom [25] respectively. However, owing to its decreased dipolar relaxation rates, which carry all the structural information, as a result of its small magnetic moment and a large line-broadening amplitude due to a long electronic relaxation time $T_{1e} \sim 10^{-9}$ s [26], n.m.r. investigation of Cu(II) complexes must be used with caution. Consequently attempts to calculate distance from the linewidths are invalid [27–29]; however, the line broadening for a specific resonance may be used to obtain qualitative structural information on whether the system is in an exchange-limited process or not [22,23,28,29]. As has been generally encountered, the large paramagnetic broadening observed in the presence of small amounts of Cu(II) ions indicates that the metal ions are effectively exchanging between the various sites. In these conditions, each resonance corresponds to the weighted average of unbonded and bonded ligand. The broadening studies rely on 'fast-exchange' conditions [27,30].

It is well known that the study of the broadening provides information about the dynamics of the exchange of peptide between its free form and the Cu(II) complex [22]. In order to elucidate the mechanism of the relaxation time, T_{2p}, and to check our above hypothesis, we studied the proton temperature-dependence of line broadening for a ligand/Cu(II) ratio of 10:1 at pH 9.40. Indeed, the effect of temperature on the observed values of T_{2p}^{-1} can be used to determine the dominant (slowest) process [25]. From the logarithm of the broadening of the C-2–H and C-5–H imidazole resonance by Cu(II) as a function of the inverse of temperature, it was found that T_{2p}^{-1} values do not change markedly with increasing temperature in the range 23–70 °C. Therefore, in this temperature region, the system is in intermediate exchange in the ^1H-n.m.r. time scale between free and bonded environments. As observed above, the absence of line broadening under strongly basic conditions reflects the strengthening of the Cu(II)-ion–peptide binding and the resulting slower dissociation, i.e. our system is in a slow exchange process, as observed earlier [22,23].

Nevertheless, at pH 9.40, where only the $MH_{-2}L$ species is present, the line broadening $\Delta\gamma_{\frac{1}{2}}$ (Hz) can be measured accurately. From their behaviour of the line

broadening we can conclude that the α-amino, the two deprotonated peptide and the imidazole nitrogen atoms are implicated in the co-ordination mode around the Cu(II). But the fact that in acidic or neutral medium the largest broadening is observed for the *N*-terminal fragment and for the imidazole ring may suggest the possibility of the presence of minor species, where complication occurs via the terminal amino group and the imidazole group, forming a bis-complex, as proposed by potentiometric titration (see above). However, this interpretation, which must be used with caution, is valid only if all the three species are exchanging at the same rate at the n.m.r. time scale.

The similarity of the ^{1}H- and ^{13}C-n.m.r. results for the TLH and the native-sequence tripeptide DAH suggests that the nature of the side chain at position 1 (Thr or Asp) and 2 (Leu or Ala) has no major effect on the formation of the Cu(II)–peptide complex, whereas the presence of the histidine residue in third position is crucial for formation of the complex. However, with DAH it was proposed that the Cu(II) complex is a pentaco-ordinate structure, consisting of four nitrogen ligands and the β-carboxy side chain on Asp [28]. More recent ^{1}H and ^{13}C-n.m.r. experiments on metal ion binding to the (1–24)-peptide fragment of HSA revealed a similar behaviour. Nevertheless, with the AFP native-sequence tripeptide we have no indication of the participation of the threonine side chain in formation of the complex. More likely we have a square-planar complex involving the four nitrogen ligands, as proposed in the model for the Cu(II)-transport site of albumin [31].

Nine superhyperfine lines in e.p.r. spectra also strongly support the co-ordination of four nitrogen atoms with Cu(II). The data show the pattern typical for nearly tetragonal symmetry ($g_{\parallel} > g_{\perp}$) having well-resolved parallel hyperfine lines and large $A_{\parallel}$ value. These $g_{\parallel}$, $g_{\perp}$ and $A_{\parallel}$ values are compatible with four-nitrogen ligation (4N) around Cu(II) [32] and are in agreement with those observed elsewhere [33].

The results obtained from equilibrium dialysis indicate that the tripeptide TLH can remove Cu(II) from the Cu(II)–albumin complex very effectively at neutral pH. At equimolar concentrations about 52% of the Cu(II) was bound to TLH (Fig. 4), compared with 44% and 72% in the case of GGH and DAH respectively [15,17]. The latter two are the model peptide (GGH) as well as the native-sequence tripeptide (DAH) for the Cu(II)-transport site of HSA. It may be noted that the stability constants of the major complex species $MH_{-2}L$ for these three tripeptides fall in the same order. The corresponding values as $\log\beta_{pqr}$ are −1.34, −1.92 and −0.55 for TLH, GGH and DAH respectively [17]. The similarity of Cu(II)-ion-binding characteristics of TLH and DAH suggests that the *N*-terminal section of AFP may be the specific Cu(II)-ion-binding site, just as for HSA, and thus serves the function of transporting Cu(II) ions in fetal life.

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