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Synthesis of the Native Copper(II)-Transport Site of Human Serum Albumin and its Copper(II)-Binding Properties

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A derivative of the native-sequence tripeptide of the specific Cu(II)-transport site of human serum albumin, L-aspartyl-L-alanyl-L-histidine *N*-methylamide, was synthesized, and its binding to Cu(II) was examined to determine the influence of the side-chain groups on the Cu(II) binding. The equilibria involved in the Cu(II)–L-aspartyl-L-alanyl-L-histidine *N*-methylamide system were investigated by analytical potentiometry. Three complex species were found in the pH range 4–10. The same species were identified in both the visible and circular-dichroism spectra. The main species present in the physiological pH range is shown to have the same ligands around the square-planar Cu(II) ion as those reported for albumin and tripeptides diglycyl-L-histidine and its *N*-methylamide derivative. The results obtained from competition experiments showed that this tripeptide has a higher affinity towards Cu(II) than has albumin itself. The overall findings are compared with those from albumin. At neutral pH the side chains do not play any important role in the Cu(II) binding, but at low pH the β-carboxyl group of the *N*-terminal aspartic residue becomes important. A possible competition site on albumin for Cu(II) at low pH is discussed.

The Cu(II)-transport site of human serum albumin involves the α-amino nitrogen of the *N*-terminal aspartic acid residue, two intervening peptide nitrogen atoms and the imidazole nitrogen of the histidine residue in the third position (Bearn & Kunkel, 1954; Peters & Blumenstock, 1967; Shearer *et al.*, 1967; Neumann & Sass-Kortsak, 1967; Bradshaw *et al.*, 1968; Sarkar & Wigfield, 1968; Bradshaw & Peters, 1969). The tripeptide diglycyl-L-histidine and its *N*-methylamide derivative, which were the simplification of the natural binding-site sequence, have been shown to have a similar binding site for Cu(II) (Lau *et al.*, 1974; Kruck *et al.*, 1976; Camerman *et al.*, 1976). However, the Cu(II)-binding strength and the Cu(II)-exchange rate (Lau & Sarkar, 1975) for both peptides differ from that of albumin. To evaluate any influence arising from the *N*-terminal aspartic acid residue and the alanine residue in the second position of the protein, the study of the native-protein *N*-terminal tripeptide sequence without the rest of the protein molecule was inevitable as the initial step. Thus the *N*-methylamide derivative of the tripeptide L-aspartyl-L-alanyl-L-histidine was chosen to approximate more closely to the protein structure. This small molecule also permits studies that often cannot be applied to large protein molecules.

In the present paper, the detailed synthesis and the Cu(II)-binding studies of the amide derivative of the native-sequence tripeptide are reported. A competition study between human albumin and the peptide for Cu(II) ion was also undertaken.

Materials and Methods

Materials

Crystalline human serum albumin obtained from Miles Laboratories (Kankakee, IL, U.S.A.) was used without further purification. The radioisotope ^{67}Cu(II) was prepared as previously described (Marceau *et al.*, 1970). Dialysis membrane was obtained from Visking Co. (c/o Union Carbide, Toronto, Ont., Canada). Potassium hydrogen phthalate was purchased from the National Bureau of Standards (Washington, DC 20234, U.S.A.). Z‡-L-Ala-OH, L-His-OMe,2HCl and γ-OBu^t-Z-L-Asp-ONSu were obtained from Fluka A.G. (Buchs, Switzerland). All other reagents were of analytical grade.

Melting points are reported uncorrected. 1,2-Dimethoxyethane was dried by refluxing over sodium and distilling. Purification of triethylamine was carried out by keeping over NaOH pellets and distilling from a mixture with 2% (w/v) phenyl iso-

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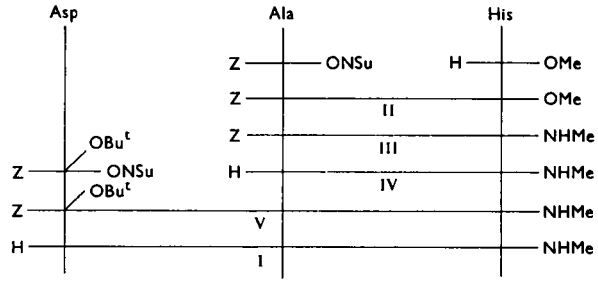
‡ Abbreviations: Z, benzyloxycarbonyl; OBu^t, tertiary butoxy; ONSu, succinimido-oxy; c.d., circular dichroism.



Scheme 1. *Synthesis scheme for* L-*aspartyl*-L-*alanyl*-L-*histidine N-methylamide* (*I*), *by the active ester method*
First stage of the synthesis is the coupling of the *N*-hydroxysuccinimide ester of *N*-benzyloxycarbonyl-L-alanine with L-histidine methyl ester to produce the protected dipeptide ester (II). For the meanings of the other symbols and coupling reactions see the text.

cyanate (Sauer, 1963). For t.l.c., precoated silica-gel plates (Eastman Chemical Co., Rochester, NY, U.S.A.) were used with the following systems: A, chloroform/methanol/acetic acid (17:2:1, by vol.); B, butan-1-ol/acetone/water/acetic acid (4:3:2:1, by vol.); C, butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.); D, ethyl acetate/pyridine/water/acetic acid (5:5:3:1, by vol.). For quantitative amino acid analysis, a sample was hydrolysed with constant-boiling HCl in a sealed ampoule at 110°C for 22h, and the ratio of amino acids was determined with a Technicon TSM instrument, by the method of Hamilton (1963). Elemental analyses were carried out by the Microanalysis Laboratory, St. George St., Toronto, Ont., Canada.

Synthesis of L-*aspartyl*-L-*alanyl*-L-*histidine N-methylamide*

The tripeptide L-Asp-L-Ala-L-His *N*-methylamide (I) was synthesized by the active ester method (Anderson *et al.*, 1964) according to the protocol shown in Scheme 1. Coupling of the *N*-hydroxysuccinimide ester of *N*-benzyloxycarbonyl-L-alanine with L-histidine methyl ester produced the dipeptide Z-L-Ala-L-His-OMe (II), which was then converted into the methylamide derivative (III) with methylamine. The Z-group on compound (III) was cleaved by hydrogenation and the resulting compound (IV) was treated with the *N*-hydroxysuccinimide ester of *N*-benzyloxycarbonyl-t-butyl-L-aspartic acid to give the tripeptide (V). Removal of the γ-amino and side-chain protecting groups on the aspartic residue followed by purification of the product by gel filtration yielded pure compound (I). The detailed procedures are given below.

(1) *N-Benzyloxycarbonyl*-L-*alanyl*-L-*histidine methyl ester* (*II*). To a stirred mixture of *N*-benzyloxycarbonyl-L-alanine-*N*-hydroxysuccinimide ester (6.4g, 20mmol) and L-histidine methyl ester dihydrochloride (4.84g, 20mmol) in anhydrous 1,2-dimethoxyethane (100ml) was added triethylamine (5.6ml, 40mmol) and the mixture stirred overnight. The solvent was removed *in vacuo*, the residue diluted with water (approx. 150ml) and the pH of the mixture adjusted to approx. 8 at 4°C; the precipitated white solid was filtered, washed with water and dried [yield 5g (67%); m.p. 162–164°C]. It was homogeneous on t.l.c. in solvent systems A and C. An analytical sample obtained by crystallization of the sample from aqueous ethanol melted at 172–173°C (Found: C, 58.41; H, 5.94; N, 15.04; Calc. for $C_{18}H_{22}O_5N$: C, 57.74; H, 5.92; N, 14.97%).

(2) *N-Benzyloxycarbonyl-β-t-butyl*-L-*aspartyl*-L-*alanyl*-L-*histidine N-methylamide* (*V*). A solution of *N*-benzyloxycarbonyl-L-alanyl-L-histidine methyl ester (II) (4.5g) in methanol (150ml) cooled in an ice bath was saturated with methylamine. The flask was tightly stoppered and left overnight. T.l.c. of the reaction mixture (solvent system B) showed the reaction to be complete. Solvent removal *in vacuo* gave *N*-benzyloxycarbonyl-L-alanyl-L-histidine *N*-methylamide (III) as an almost white solid. This was dissolved in methanol (50ml), and acetic acid (3ml) and 10% Pd on C (300mg) were added and the mixture was hydrogenated for 5h. The catalyst was filtered and the solvent removed *in vacuo*. Methanol was added to the residue and evaporated. This was repeated three times. The resulting gummy material on trituration with anhydrous ether solidified to give L-alanyl-L-histidine *N*-methylamide diacetate (IV). It was filtered, washed with ether and dried (yield, 3g).

A mixture of the crude compound (IV) (2.35g, 6.5mmol) and the *N*-hydroxysuccinimide ester of *N*-benzyloxycarbonyl-β-t-butyl-L-aspartic acid (2.69g, 6.5mmol) in anhydrous 1,2-dimethoxyethane (40ml) was treated with triethylamine (1.82ml, 13mmol) and the mixture stirred overnight. After solvent removal *in vacuo*, the residue was diluted with water and then extracted with 4×75–100ml of ethyl

acetate. The combined extracts were washed with water once, ice-cold 1 M-NaOH twice, saturated NaCl solution twice, dried over anhydrous Na_2SO_4, and solvent was removed to give a white solid (V) [yield 2.5 g (65%), m.p. 145–150°C]. It was homogeneous on t.l.c. in solvent systems A and B. An analytical sample was obtained by crystallizing the solid from ethyl acetate/ether (Found: C, 56.89; H, 6.73; N, 15.38; Calc. for $C_{26}H_{36}N_6O_7$: C, 57.32; H, 6.67; N, 15.44%).

(3) L-*Aspartyl*-L-*alanyl*-L-*histidine N-methylamide* (*I*). *N*-Benzyloxycarbonyl-β-t-butyl-L-aspartyl-L-alanyl-L-histidine *N*-methylamide (2.3 g), dissolved in methanol (50 ml) and acetic acid (1 ml), was hydrogenated over 10% Pd on C (200 mg) for 6 h. The crude product obtained after the usual work-up was cooled in an ice bath and treated with anhydrous HCl in 1,2-dimethoxyethane (25 ml, 6.5 M). After a few minutes, the ice bath was removed and the mixture stirred for 2 h at room temperature (20–22°C). Anhydrous ether (200 ml) was added and the precipitated solid filtered, washed with anhydrous ether and dried *in vacuo* over KOH. The solid was chromatographed on a column (2 cm × 100 cm) of Sephadex G-10 in water, and the fractions containing the pure tripeptide were pooled and freeze-dried [yield 0.8 g (50%)]. The compound showed a single spot on t.l.c. in solvents C and D. Amino acid analysis of the hydrolysate of this material gave Asp/Ala/His as 0.95:1.04:0.96 (Found C, 42.25; H, 6.39; Cl, 9.51; N, 21.41. Calc. for $C_{14}H_{23}ClN_6O_5$: C, 42.90; H, 5.88; Cl, 9.08; N, 21.49%).

Potentiometric titration

The titrations were carried out on a Radiometer automatic titration assembly thermostatically maintained at 25°C (Lau *et al.*, 1974). NaOH, prepared carbonate-free and kept under argon atmosphere, was standardized against primary standard potassium hydrogen phthalate. $CuCl_2$ was dissolved in 1 mM-HCl to give a stock solution of 0.1044 M and standardized complexometrically against EDTA, with murexide as indicator. The peptide stock solution was prepared and stored at 4°C when not in use. All the solutions contained known amounts of HCl to lower the starting pH below that of metal binding and were then titrated with 0.1090 M-NaOH in the presence and absence of Cu(II) from pH 2.5 to 10.5.

Spectrophotometry

The visible absorptions of the Cu(II) complexes were measured on a Cary model 15 spectrophotometer with a 5 cm light-path, and the cell compartment was thermostatically maintained at 25°C. The c.d. spectra initially were recorded on a Rousell-Jouan Dichographe II (S. A. Jouan, Paris, France) with a maximum wavelength of 610 nm, and then on a Jasco ORD/UV-5 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) with a c.d. attachment that extended the range to 700 nm. Spectra as a function of pH were measured at 20–22°C, at a 1:1 Cu(II)/ligand ratio with concentrations in the 1–10 mM range in 0.15 M-NaCl solutions.

Equilibrium dialysis

The competition for Cu(II) between the native-sequence tripeptide amide and albumin was carried out in 0.1 M-*N*-ethylmorpholine/HCl buffer, pH 7.53, at 6°C and I 0.16 by the equilibrium-dialysis technique described by Lau & Sarkar (1971). One half-cell contained the stock solution of Cu(II)–albumin mixed with $^{67}CuCl_2$, the other half-cell contained various known amounts of the peptide. The molar ratio of the peptide/Cu(II)–albumin ranged from 0.1 to 1.0. Radioactivity of $^{67}Cu(II)$ was measured before and after dialysis.

Results

Determination of species distribution and stability constants

The complexation reactions occurring between C_M mol of metal ion M, C_H mol of hydrogen H and C_A mol of ligand anion A can be represented by the general equilibrium reaction:

$$pM + qH + rA \rightleftharpoons M_pH_qA_r \qquad (1)$$

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

$$\beta_{pqr} = \frac{M_pH_qA_r}{m^p h^q a^r} \qquad (2)$$

where m, h and a are the concentrations of free metal ion, hydrogen ion and ligand respectively. The following sets of equations define the total system:

$$C_M = m + \sum p\beta_{pqr} m^p h^q a^r \qquad (3)$$

$$C_H = h - oh + \sum q\beta_{pqr} m^p h^q a^r \qquad (4)$$

$$C_A = a + \sum r\beta_{pqr} m^p h^q a^r \qquad (5)$$

where oh represents the amount of free hydroxyl ions. The experimental data and titration curves [$-\log h$ = f (base)] were obtained from solutions containing different concentrations of C_M, C_H and C_A.

The following relationships were used to obtain values for the unbound portions of metal and ligand throughout the titration:

$$pM = pM_0 + \int_{pH_0}^{pH_i} \frac{\delta H_l^+}{\delta C_M} dpH \quad (6)$$

$$pA = pA_0 + \int_{pH_0}^{pH_i} \frac{\delta H_l^+}{\delta C_A} dpH \quad (7)$$

where pM = −log[free metal M], pA = −log[free ligand A], pH = $-\log h$, H_l^+ = mol of OH^- consumed in the titration of the hydrogen ions liberated from the complexation reactions. Subscript 0 denotes the initial (known) system. The mathematical analyses of the data were performed by the sequential use of three computer programs* by using a GE 440 computer according to the method described earlier (Sarkar & Kruck, 1973). The program PLOT-2 takes the numerical titration data and solves eqns. (6) and (7) to give, from metal-variation data, the proton-liberation term $\delta H_l^+/\delta C_M$ and the amounts of free metal ion at each selected pH value; likewise, from ligand-variation data $\delta H_l^+/\delta C_A$ the free-ligand concentrations are obtained. A subroutine is incorporated which for polyfunctional weak acids determines the amount of fully deprotonated ligand. The program GUESS-2, using the data from PLOT-2, sets up a matrix of the terms $m^p h^q a^r$ (eqn. 2) for each proposed species at each selected pH value. This matrix then serves as an input to the program LEASK-2, which uses an iterative least-squares minimization procedure to calculate the stability constants, β_{pqr}. For a fuller description see Lau *et al.* (1974) and Sarkar & Kruck (1973).

(*a*) *Proton-L-aspartyl-L-alanyl-L-histidine N-methylamide system* (*H*:*A*). Solutions 1–3 in Table 1 were titrated. Since the buffering region of the third dissociable proton was more than 2 pH units away from that of the next proton, the proton-liberation data for the third proton was treated with the Henderson–Hasselbach equation:

$$pH = pK_a + \log\frac{[base]}{[acid]} \quad (8)$$

The two overlapping buffering regions were processed by the programs outlined above to yield the protonation constants β_{011} and β_{021} of the species HA and H_2A (where A is the anionic form of L-aspartyl-L-alanyl-L-histidine *N*-methylamide). The refined values of $\log\beta_{0ql}$ for the successive protonated species are listed in Table 2.

* These programs (PLOT-2, GUESS-2 and LEASK-2) are available in both FORTRAN-IV and APL languages. All enquiries about these programs should be directed to B. Sarkar.

Table 1. *Sample composition of the systems titrated in* 0.15M-*NaCl at* 25°C

Sample no. Proton–ligand system (H:A)	Total amounts (μmol) Metal	Ligand
1	—	21.65
2	—	32.48
3	—	43.30
Metal–ligand system (M:H:A) Metal variation		
4	5.22	43.30
5	10.44	43.30
6	20.88	43.30
Ligand variation		
7	10.44	21.65
8	10.44	32.48
9	10.44	43.30

Table 2. *Comparison of log(stability constants)* ($\log\beta_{pqr}$) *of the complex species* $M_pH_qA_r$ (*M* = *Cu(II)*, *A* = *tripeptide*) *in* 0.15M-*NaCl at* 25°C

The tripepetides are: A, L-aspartyl-L-alanyl-L-histidine *N*-methylamide; A′, diglycyl-L-histidine (Lau *et al.*, 1974); A″, diglycyl-L-histidine *N*-methylamide (Kruck *et al.*, 1976).

p	*q*	*r*	$\log\beta_{pqr}$(A)	$\log\beta'_{pqr}$(A′)	$\log\beta''_{pqr}$(A″)
0	3	1	17.27	17.50	—
0	2	1	14.29	14.78	14.47
0	1	1	7.73	8.04	8.00
1	0	1	8.39	9.220	—
1	−1	1	—	3.648	—
1	−1	2	10.01	—	—
1	−2	1	−0.55	−1.991	−0.479

(*b*) *Cu(II)–proton–L-aspartyl-L-alanyl-L-histidine N-methylamide system* (*M*:*H*:*A*). In the metal-variation experiments, solutions 4–6 (Table 1) were titrated, and, in the ligand-variation experiments, solutions 7–9 (Table 1) were titrated. The data were processed by program PLOT-2 to yield pM, pA, $\delta H_l^+/\delta C_M$ (Fig. 1, curve 1) and $\delta H_l^+/\delta C_A$ (Fig. 1, curve 2) as a function of pH. For the species selection of the complexes formed in the metal–ligand system, the following values for *p*, *q* and *r* were used: $p = 1$; $q = +2, +1, 0, -1, -2, -3$; $r = 1, 2$. The intermediate data pM and pA as a function of pH were further processed by programs GUESS-2 and LEASK-2. The calculations indicated that the complex species MA, $MH_{-2}A$ and $MH_{-1}A_2$* were

* For the meaning of the species $MH_{-2}A$ and $MH_{-1}A_2$ see the Discussion section.

required to give a minimum-error solution. The results for the stability constants expressed as $\log\beta_{pqr}$ are listed in Table 2, and the species distribution as a function of pH is shown in Fig. 2.

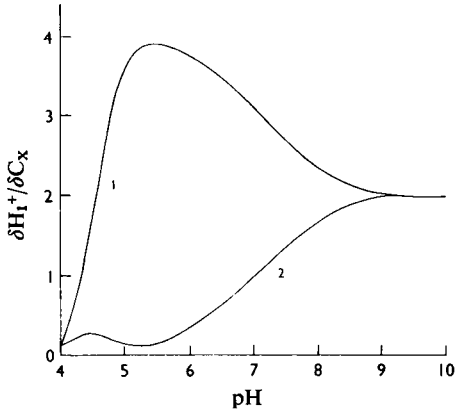


Fig. 1. *Proton liberation as a function of pH for the system Cu(II)–L-aspartyl-L-alanyl-L-histidine N-methylamide*
Curve 1, $\delta H_1^+/\delta C_M$; curve 2, $\delta H_1^+/\delta C_A$ (see the text for meaning and implication).

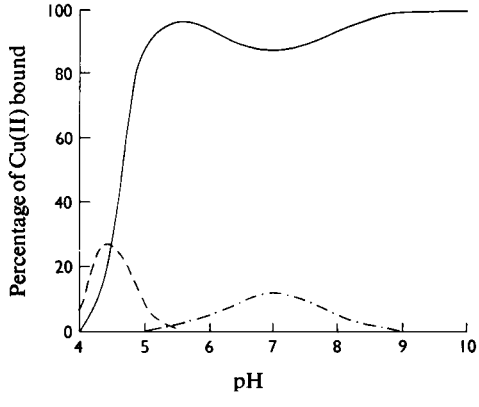


Fig. 2. *Species distribution as a function of pH for the system Cu(II)–L-aspartyl-L-alanyl-L-histidine N-methylamide*
----, MA; ——, $MH_{-2}A$; -·-·, $MH_{-1}A_2$.

Visible-absorption spectra

A solution of equimolar concentration of Cu(II) and the peptide (1.982M) was prepared in 0.15M-NaCl, and the visible-absorption spectra were measured at various pH values. The spectrum obtained above pH10 arises solely from the species $MH_{-2}A$, which has the spectral characteristics of $\lambda_{max.} = 525$nm and $\varepsilon_{max.} = 103$. The spectral data for various pH values together with that of the species $MH_{-2}A$ were processed by the method described earlier (Kruck & Sarkar, 1975) to yield the calculated spectra for individual species. The results are shown in Fig. 3. The spectral characteristics of the species are compared in Table 3.

C.d. spectra

The c.d. spectra of Cu(II)–L-aspartyl-L-alanyl-L-histidine *N*-methylamide were obtained as a function of pH (Fig. 4). In the pH region 3.9–5.0, a single Cotton effect was observed under the 710nm-absorption band which reached a maximum intensity around pH4.5. This must arise from the species MA observed in the potentiometric–spectroscopic analysis. Beyond pH4.7 two Cotton effects are observed at 565nm (negative) and 485nm (positive); these reached

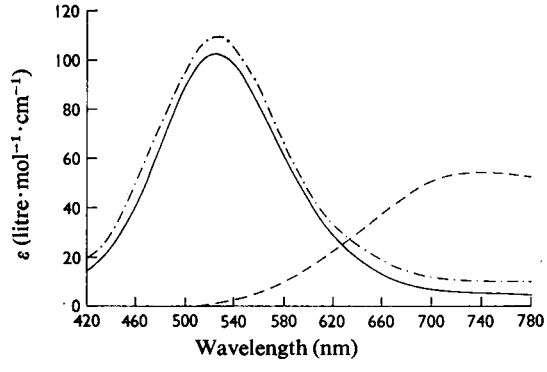


Fig. 3. *Computed visible-absorption spectra of the species in the system Cu(II)–L-aspartyl-L-alanyl-L-histidine N-methylamide, at 25°C and I 0.16*
----, MA; ——, $MH_{-2}A$; -·-·, $MH_{-1}A_2$.

Table 3. *Comparison of spectral characteristics of the Cu(II) complexes in* 0.15M-*NaCl at 25°C*

Cu(II) complex	$\lambda_{max.}$ (nm)	$\varepsilon_{max.}$ (litre·mol^{-1}·cm^{-1})
Human albumin–Cu(II)*	525	101
Gly-Gly-L-His–Cu(II) ($MH_{-2}A$)*	525	103
Gly-Gly-L-His-NHMe–Cu(II) ($MH_{-2}A$*)	525	103
L-Asp-L-Ala-L-His-NHMe–Cu(II) ($MH_{-2}A$)	525	103
L-Asp-L-Ala-L-His-NHMe–Cu(II) ($MH_{-1}A_2$)	527	110
L-Asp-L-Ala-L-His-NHMe–Cu(II) (MA)	730	55

* Values taken from Kruck *et al.* (1976).

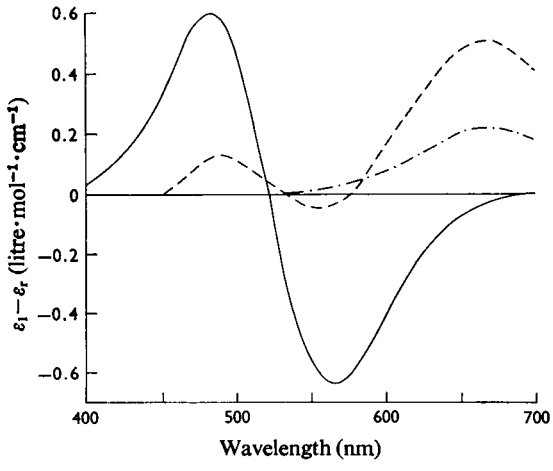


Fig. 4. *Circular-dichroism spectra of Cu(II)-L-aspartyl-L-alanyl-L-histidine N-methylamide solutions, at* 20–22°C, *I* 0.16 *and a* 1:1 *metal/peptide ratio*
–·–·, pH3.90; ----, pH4.30; ——, pH7.0–8.4.

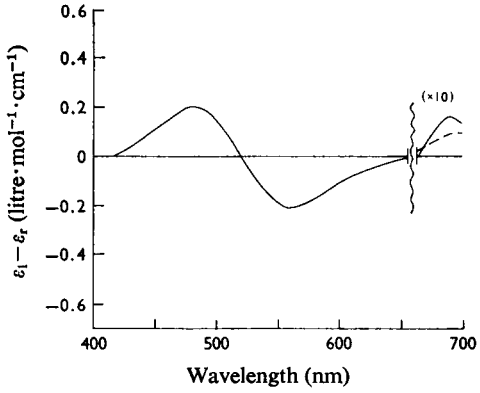


Fig. 5. *Circular-dichroism spectra of Cu(II)–human serum albumin solutions, at* 20–22°C, *I* 0.16 *and a* 1:1 *metal/protein ratio*
----, pH4.30; ——, pH7.40.

a maximum at neutral pH and remained constant over the range pH7–9. These two transitions can be related to the dominant 1:1 species from the potentiometric–spectroscopic analysis. Under the conditions used in obtaining the c.d. spectra, i.e. at a 1:1 ratio of metal/ligand, any bis-chelated species would be present in a negligible amount and was certainly not observed in these spectra.

The c.d. spectra of Cu(II)–albumin are shown in Fig. 5. At low pH(4–4.5) a *d–d* absorption band is observed at 690nm (ε approx. 60); associated with this band is a weak but measurable Cotton effect above 660nm (in general, the magnitude of the Cotton effects is weaker for the albumin complex than for the peptide complexes). In the neutral-pH region, two Cotton effects are observed of the same sign and at the same wavelength as found for the tripeptide complex.

Comparison of Cu(II)-binding strength

Competition for Cu(II) between albumin and the native-sequence tripeptide amide was carried out by the equilibrium-dialysis technique, with ^{67}Cu(II) as tracer. The dissociation of the Cu(II)–peptide complex and the competition reaction are similar to that expressed previously (Lau *et al.*, 1974). The calculated equilibrium concentrations and the dissociation constant are presented in Table 4. The average value of the dissociation constant K_D [Cu(II)–peptide] is 1.099×10^{-17}M.

Discussion

The tripeptide L-aspartyl-L-alanyl-L-histidine *N*-methylamide has three titratable protons in the pH range studied (2.5–10.5). These can be attributed to the β-carboxyl, the imidazole and the amino groups with pK_a values of 2.98, 6.55 and 7.73 respectively. The tripeptide starts to bind Cu(II) at a pH slightly below 4, after which there is a rapid proton displacement (Fig. 1) rising to a maximum displacement of about four protons in a range of less than 2pH units. This rapid displacement can be attributed to the formation of the major species $MH_{-2}A$, [more than

Table 4. *Equilibrium-dialysis data of the ternary binding system*
Equilibrium dialysis was carried out in 0.1M-*N*-ethylmorpholine/HCl buffer, pH7.53, 6°C, *I*0.16. Total initial Cu(II)–albumin concentration was kept at 300μM while the peptide concentration was varied. The complex species $MH_{-1}A_2$ was ignored in the calculation. All concentrations are given as μM.

[Total peptide]	[Cu(II)–peptide]	[Peptide]	[Cu(II)–albumin]	[Albumin]	$K_{eq.}$*	K_D, Cu(II)–peptide*/10^{17}M
29	2.83	0.7	271.7	28.3	4.21	1.570
76	72.6	0.34	227.3	72.7	6.82	0.969
152	131.8	20.2	168.1	131.9	5.12	1.291
228	187.5	40.5	112.5	187.5	7.72	0.856
285	214.6	70.3	85.4	214.6	7.66	0.863

* Defined in Lau *et al.* (1974).

88% of total Cu(II)] above pH5.5. Since the β-carboxyl group is already titrated, the four protons can be attributed to those from the imidazole, amino and the two peptide amide groups. The proposed structure of this species (Fig. 6), in which the amino, imidazole and two peptide nitrogen atoms co-ordinate to the central Cu(II) ion to form a square-planar configuration, is the same as that proposed for the major 1:1 species of the Cu(II) complexes of albumin and the tripeptides diglycyl-L-histidine and diglycyl-L-histidine *N*-methylamide at physiological pH (Peters & Blumenstock, 1967; Lau *et al.*, 1974), and the same as that found in the solid state of the Cu(II) complex of the latter peptide obtained by crystallization from neutral solution (Camerman *et al.*, 1976). Further confirmation of the structure is obtained from identical spectroscopic characteristics of the above complexes (Tables 3 and 4). In the pH region 5–9, the proton-displacement results indicate the presence of another species, apart from the major species, $MH_{-2}A$, which reaches a maximum concentration of 12% of the total Cu(II) bound at pH7. The visible-absorption spectra are also consistent with the presence of another species with characteristics of $\lambda_{max.} = 527$ nm and $\varepsilon_{max.} = 110$, over the same pH range. For minimum-error solution of the proton-displacement data the stoicheiometry of this species corresponds to a bis-chelated form $MH_{-1}A_2$. This species was not found in the other two tripeptide systems (Lau *et al.*, 1974; Kruck *et al.*, 1976). However, it is noteworthy that Cu(II) can induce aggregation of human serum albumin (Österberg *et al.*, 1975). Below pH5, another species, MA, is detected, which reaches a maximum of about 60% of the total bound form at pH4.45. This species has a broad absorption maxima at approx. 730nm ($\varepsilon_{max.} = 55$) and gives rise to a single positive Cotton effect with a maximum differential absorption at 670nm. The pH-dependence and spectroscopic characteristics are consistent with MA, having one nitrogen and three oxygen atoms bound to Cu(II) (Freeman, 1967), which most likely involves the terminal amino and β-carboxyl groups (Scheme 2). In the intermediate pH range 5–6, Cu(II)–albumin exhibits three distinct *d–d* absorption bands at 525, 660 and 730nm (Appleton & Sarkar, 1971; Breslow, 1964). Based on this observation is the suggestion that there are competitive sites on albumin available for Cu(II) binding at low pH (Breslow, 1964). The results presented here may clarify this point further.

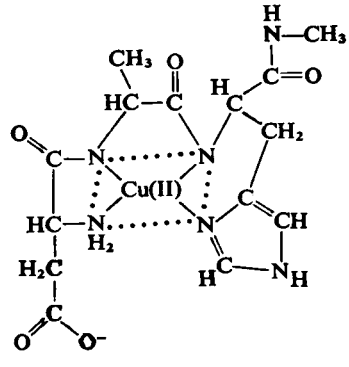
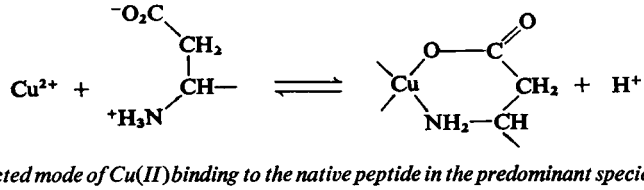


Fig. 6. *Proposed structure of the main Cu(II)–L-aspartyl-L-alanyl-L-histidine N-methylamide species ($MH_{-2}A$) in the physiological pH range*

The 730nm band corresponds to the low-pH species MA observed in the Cu(II)–native-peptide system involving the *N*-terminal and β-carboxyl groups; the same binding mode is also evident in the Cu(II)–albumin system at low pH. The 525nm band is the major 1:1 species discussed above and is of stoicheiometry $MH_{-2}A$ in the Cu(II)–native-peptide complex. A 660nm band is not observed in the Cu(II)–native-peptide system. There are two possible explanations for this finding. The 660nm band may arise from a competitive site on albumin for Cu(II) binding at low pH. This site may not necessarily be located at the *N*-terminus. Alternatively, the 660nm band is at a wavelength expected for the unobserved $MH_{-1}A$ species in the peptide system. It is possible that the species may be a transient intermediate in the formation of $MH_{-2}A$ from the starting MA in the Cu(II)–peptide system. For Cu(II)–albumin, the final deprotonation step converting $MH_{-1}A$ into $MH_{-2}A$ may require some conformational change, so that $MH_{-1}A$ becomes observable. Further elucidation of this mechanism would require a kinetic study on the formation of the Cu(II)–albumin complex.

Circular dichroism, by its nature, is known to be a more sensitive indicator of a metal-ion environment



Scheme 2. *Depicted mode of Cu(II) binding to the native peptide in the predominant species (MA) at low pH*

Table 5. *C.d. extrema and absorption maxima of the Cu(II)–complexes in* 0.15 M-*NaCl at* 25°C

pH	$\lambda_{max.}$ (ε litre·mol^{-1}·cm^{-1}) (nm)	c.d. maxima ($\varepsilon_l - \varepsilon_r$ litre·mol^{-1}·cm^{-1}) (nm)
	L-Asp-L-Ala-L-His-NHMe–Cu(II)	
4.3	730 (40), 520 (sh.)*	670 (+0.51), 555 (–0.05), 492 (+0.13)
7.4	†	565 (–0.64), 485 (+0.57)
	L-Asp-L-Thr-L-His-L-Lys-O$^-$–Cu(II)‡	
7.0	530 (98)	565 (–0.70), 485 (+0.51)
	Human serum albumin–Cu(II)	
4.3	690 (~60)	>700 (+)
7.4	†	565 (–0.21), 485 (+0.24)
	Bovine serum albumin–Cu(II)	
7.0	530 (93)	560 (–0.73), 480 (+0.27)

* Shoulder,
† See data in Table 3.
‡ Data taken from Bradshaw *et al.* (1968).

than the *d–d* electronic absorption spectrum, and under favourable circumstances allows comment on conformational aspects of the ligand bound to the metal. To our knowledge, this is the first time that such spectra have been related directly to known species in a kinetically labile multiple-species system. Thus, in Cu(II)–L-aspartyl-L-alanyl-L-histidine *N*-methylamide, the three Cotton effects observed at pH4.3 and 4.7 can be attributed to the presence of the species MA and $MH_{-2}A$ in solution, the longer-wavelength transition arising from the species MA, which reaches a maximum concentration at pH4.5, and the other two Cotton effects arising from the $MH_{-2}A$ species. The close similarity of the spectroscopic parameters (Table 5) of the 1:1 species of the Cu(II)–native-peptide and Cu(II)–albumin complexes, both having two Cotton effects of the same sign at the same wavelengths and cross-over points at 525nm, is further conclusive evidence that the peptide complexes duplicate the specific Cu(II)–albumin-binding mode.

Bradshaw *et al.* (1968) have compared the c.d. spectra of the Cu(II) complexes of the tetrapeptide L-Asp-L-Thr-L-His-L-Lys-O$^-$, bovine serum albumin and the 1–24 *N*-terminal peptide unit of bovine albumin. The c.d. spectrum of the Cu(II)–human albumin complex at neutral pH is similar to that reported by Bradshaw *et al.* (1968) for the Cu(II)–bovine albumin complex, except that their 560nm transition has a differential molar absorption of –0.73, whereas we find a value of only –0.21. This latter value was observed with human protein from two different reliable commercial sources and on two different instruments. This difference may well arise from the species difference between the two albumins. The c.d. spectra of Cu(II) complexes of their tetrapeptide and the 1–24 peptide unit are, except for small differences in $\Delta\varepsilon$ values, identical with that of the 1:1 Cu(II)–L-aspartyl-L-alanyl-L-histidine *N*-methylamide complex reported here. Spectrally, this indicates that no significant influence is exerted either by the substitution of threonine for alanine in the second position or from any of the residues after position 3 on the metal-binding site, again substantiating the previous conclusion that only the first three amino acid residues are involved in the binding of the Cu(II) ion. The present findings indicate that, although the native tripeptide structurally resembles the specific Cu(II)-transport site of albumin, there still exist subtle differences in the binding strength and exchange properties. As shown in Table 2, the two tripeptide amides have comparable stability, whereas the peptide diglycyl-L-histidine is less stable by about 30-fold. The results obtained from equilibrium dialysis indicate that the native tripeptide exhibits about 5–6-fold higher binding affinity for Cu(II) than does albumin. This is also consistent with the observation of the ligand-exchange rate for the same peptide. The Cu(II)-exchange rate between L-histidine and L-aspartyl-L-alanyl-L-histidine *N*-methylamide was 2.70s^{-1}, which is 4 times that found for albumin under the same experimental conditions (S. Lau & B. Sarkar, unpublished work). The observed differences could well be ascribed to the influence of the neighbouring amino acids (lysine, serine etc.), which could affect the local conformation to a certain extent, or the solvation property of the ligands, or both.

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