A Peptide Molecule Mimicking the Copper(II) Transport Site of Human Serum Albumin

A COMPARATIVE STUDY BETWEEN THE SYNTHETIC SITE AND ALBUMIN*

(Summary)

Diglycyl-L-histidine is a peptide molecule designed to mimic the specific Cu(II) transport site of human albumin. The equilibria involved in the Cu(II)-diglycyl-L-histidine system have been investigated by analytical potentiometry in aqueous solution (0.15 M NaCl, 25°C). The synthetic peptide molecule bound Cu(II) exclusively as a 1:1 complex in the pH range of 6.50 to 11.00. The results further showed that the complex has the same ligand atoms binding to Cu(II) as those suggested for the specific Cu(II) binding site of human albumin. The interaction of peptide with Cu(II) in the presence of albumin, studied by equilibrium dialysis at pH 7.53 and ionic strength 0.16, indicates that this tripeptide is able to compete with albumin for Cu(II). The dissociation constant of the Cu(II)-peptide complex has a value of 1.18 x 10^-16 as compared with 6.61 x 10^-17 for Cu(II)-albumin. The lower Cu(II) binding strength of the peptide may reflect the influence of either the COOH-terminal free carboxyl group of the peptide or the side chain residues of the Cu(II) binding site in the native protein or both. In the presence of equimolar concentrations of albumin and peptide, and approximately 20-fold excess of L-histidine, there is about 18% of Cu(II) present in the forms of Cu(II)-albumin and L-histidine-Cu(II)-albumin, 36% in the forms of Cu(II)-peptide and L-histidine-Cu(II) peptide, and 46% as Cu(II)-L-histidine. The biomedical implications of designing a small molecule to mimic certain functions of a biologically important macromolecule are discussed.

Human serum albumin has one specific Cu(II) binding site (2, 3). Cu(II) bound to albumin is considered to be the transport form of Cu(II) in blood (2, 4). The characterization of this site has been possible because of careful investigations from the laboratories of Peters (5-9), Breslow (10), and Gurd (9, 11). The Cu(II) binding site has been shown to involve the α-amino nitrogen of the NH2-terminal aspartic acid residue, two intervening peptide nitrogens and the imidazole nitrogen of the histidine residue in position 3 (7, 9, 11). This laboratory has been engaged in applying the molecular design concept to metal binding sites of proteins for some time (12-15). The well defined Cu(II) binding site of albumin provided an ideal opportunity to explore the design aspect of this site. Considering the amino acid sequences at the metal binding site of albumin from human (8), rat (8), bovine (9), and dog (16, 17) as well as the geometry and the nature of the Cu(II) binding site, a peptide, diglycyl-L-histidine, was chosen to mimic the native Cu(II) transport site of albumin (12, 13). This represents a simplification of the

LIST OF SYMBOLS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Meaning</th>
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<tbody>
<tr>
<td>M, H, A</td>
<td>Cu(II) ion, hydrogen ion and anionic form of diglycyl-L-histidine</td>
</tr>
<tr>
<td>H_+</td>
<td>Protons being titrated per mole of ligand or metal</td>
</tr>
<tr>
<td>m, h, a, oh</td>
<td>Concentrations of free Cu(II) ion, proton, anionic form of diglycyl-L-histidine and hydroxyl ion expressed in moles/liter</td>
</tr>
<tr>
<td>p, q, r</td>
<td>Number of M, H, A</td>
</tr>
<tr>
<td>β_{ppr}</td>
<td>Stoichiometric stability constant of the complex species M_pH_qA_r</td>
</tr>
<tr>
<td>[M_pH_qA_r]</td>
<td>Concentration of the species M_pH_qA_r</td>
</tr>
<tr>
<td>pX = -log x</td>
<td>e.g. pH = -log h, pM = -log m</td>
</tr>
<tr>
<td>S.D. (β_{ppr})</td>
<td>Estimated standard deviation of β_{ppr}</td>
</tr>
<tr>
<td>A</td>
<td>Absorbance of the solution</td>
</tr>
<tr>
<td>M</td>
<td>Number of rows in the matrix</td>
</tr>
<tr>
<td>N</td>
<td>Number of columns in the matrix</td>
</tr>
</tbody>
</table>
natural binding site sequences. Interestingly enough, this is one of the histidine-containing peptides which was studied, although not in detail, by Gurd and coworkers (18, 19) in order to understand the nature of the interaction of Cu(II) with myoglobin and apomyoglobin. Another histidine-containing tripeptide, glycyln-L-histidylglycine, has been shown by Österberg and Sjöberg (20, 21) to form polynuclear complexes with completely different structure and characteristics upon binding to Cu(II) in both solution and solid state. The binding of diglycyl-L-histidine with Cu(II) ion, studied by Bryce and Gurd (18), has also been shown to possess different characteristics.

In the present communication, we have made a detailed investigation of the interaction of Cu(II) with the peptide designed to mimic the Cu(II) transport site of albumin. The existing species in this system were detected by the mathematical analysis of potentiometric data and their stability constants were computed utilizing the technique of analytical potentiometry (22). Results show that one major species exists in the Cu(II)-diglycyl-L-histidine system. Structural studies show that it has the same ligand atoms binding to Cu(II) as those suggested for Cu(II)-albumin. Equilibrium studies were also undertaken to gain information as to how strongly the peptide bound Cu(II) as compared with albumin under similar experimental conditions and what was the equilibrium distribution of Cu(II) among all the Cu(II) binding species in the presence of the peptide at the conditions analogous to the physiological state. The biomedical implication of the findings is discussed.

**EXPERIMENTAL PROCEDURE**

**Materials**—Crystalline human albumin was obtained from Hoechst Pharmaceutical Company. The peptide was synthesized in our laboratory. The CBZ-diglycine p-nitrophenyl ester and L-histidine free base ([OH] = 39.4° ± 0.5°) were obtained from Nutritional Biochemicals. L-[14C]Histidine was obtained from Schwarz BioResearch. The radioisootope, 44Cu(II), specific activity at least 0.5 mCi per µg of Cu(II), was prepared (23) by irradiation of natural zinc with bremsstrahlung from the linear accelerator at the University of Toronto. Dialysis membrane was obtained from Visking Company. All other reagents were of analytical grade and the water was deionized.

**Synthesis of Diglycyl-L-histidine**—The peptide was synthesized by the active ester method (24). CBZ-diglycine p-nitrophenyl ester was reacted with L-histidine free base in equal molar ratio in 50% dioxane-water over a steam bath for 30 min. The reaction mixture was then dried under reduced pressure, and fractionated diglycyl-n-histidine were pooled and dried. These were then irradiation of natural zinc with bremsstrahlung from the linear cyl-L-histidine system. Structural studies show that it has the conditions analogous to the physiological state. The biomedical same ligand atoms binding to Cu(II) as those suggested for Cu(II) binding species in the presence of the peptide at the equilibrium-was obtained by using an automatic shaker for 6 days at 25°C ± 0.1°C. The apparatus was also standardized in terms of hydrogen ion concentration by titrating several solutions of accurately known concentrations of HCl in 0.15 mol NaCl with standard base.

**Potentiometry**—Argon was used throughout the titration to maintain an oxygen-free atmosphere. The base used for the titration a carbonate-free 1.0024 mol NaOH which was standardized against weighed amounts of primary standard potassium hydrogen phthalate obtained from the National Bureau of Standards. A CO2-free atmosphere for the base was assured at all times. A solution of 0.1 mol HCl was prepared by dilution and standardized against the calibrated NaOH. Stock solutions of CuCl2 were prepared in 10-3 mol HCl and dilutritrimetrically.

**Spectrophotometry**—Absorbance measurements were made against appropriate references in cells having 1 cm path length in a Cary model 15 recording spectrophotometer thermostated at 25°C ± 0.1°C.

**Equilibrium Dialysis**—The competitions of Cu(II) between albumin and peptide both in the presence and absence of L-histidine, were carried out by the equilibrium dialysis technique in 0.1 M N-ethylmorpholine-HCl buffer at pH 7.58 and ionic strength 0.16. The dialysis cells were the same as described previously (26). In the ternary system: one half-cell contained stock solution of Cu(II)-albumin mixed with 44CuCl2; the other half-cell contained various known amounts of peptide. The molar ratio of peptide to Cu(II)-albumin ranged from 0.2 to 20.0. In the quaternary system: one half-cell contained the same 44Cu(II)-albumin solution as mentioned above; the other half-cell contained various known amounts of peptide and constant amount of L-histidine mixed with L-[14C]histidine. The total molar ratio of L-histidine to Cu(II)-albumin was kept approximately 20 and that of peptide to Cu(II)-albumin ranged from 0.5 to 20.0. Equilibrium was obtained by using an automatic shaker for 6 days at 6°C. Radioactivity of 44Cu(II) and L-[14C]histidine was measured both before and after dialysis as described previously (26).

**CALCULATION OF RESULTS**

**Determination of Stability Constants**—The complexation reactions occurring between the species M, II, and A can be represented by the following general equilibrium reaction

\[ M + aH + bA \rightleftharpoons M_{a}H_{b}A \]  

(1)

The stabilities of the species formed are measured by the stoichiometric equilibrium constants $K_{aq}$, expressed in terms of concentrations at constant ionic strength, temperature and pressure

\[ K_{aq} = \frac{[M_{a}H_{b}A]}{[M][A]^{b}[H]^{a}} \]  

(2)

The following sets of equations define the total system

\[ C_{M} = c + a + b \]  

(3)

\[ C_{H} = h + c + b \]  

(4)

\[ C_{A} = a + b + c \]  

(5)

The experimental data and titration curves (log $h = f(base)$) were obtained from solutions of defined concentrations of $C_{M}$, $C_{H}$, and $C_{A}$. The abbreviation used is: CBZ, benzoyloxycarbonyl group. The synthesis of this tripeptide has to be carried out in a completely metal-free system. Any trace of metal contamination will cause difficulties in obtaining pure products.
and CA. The relationships which were derived by us earlier (22) were used to obtain the values for the unbound portions of metal and ligand throughout the titration. The mathematical analyses of the data were performed by the sequential use of three computer programs using a GE 440 computer.

Proton-Diglycyl-L-histidine System (H, A)—In this system p = 0, r = 1, and q = −2, −1, 0, 1, 2, and 3. A maximum of three protons can be liberated from this peptide upon titration with strong base in the pH range 1 to 11. If the pKₐ values of the different buffering groups are separated by more than two units, then the Henderson-Hasselbach equation can be applied to the titration data directly. However, in diglycyl-L-histidine, the buffering regions of two protonated groups are overlapping, the above technique cannot be used. We have solved the problem by setting up and solving a system of simultaneous equations in two unknowns.

Starting from the following definition

\[
\tilde{n}_H = \frac{\frac{Q}{\beta_0} \ln q}{\frac{\beta_0}{\beta_0 \ln q}}
\]

and rearranging Equation 6 and setting the upper limit of overlapping buffering regions to two (Q = 2), we obtain

\[
\tilde{n}_H = \frac{\beta_1 h + 2 \beta_2 h^2}{1 + \beta_1 h + \beta_2 h^2}
\]

From this we get

\[
\tilde{n}_H = \frac{\beta_1 (1 - \tilde{n}_H) h + \beta_2 (2 - \tilde{n}_H) h^2}{1 + \beta_1 h + \beta_2 h^2}
\]

where \(\tilde{n}_H\) and \(h\) are experimental data obtained from a series of titration curves which differ only in their total ligand concentration. As previously stated (22), it can be shown

\[
\tilde{n}_H = 0 - \frac{5h_i^+}{\delta C_A}
\]

Now, inserting Equation 9 into Equation 8 we get for the case of \(Q = 2\)

\[
(2 - \frac{5h_i^+}{\delta C_A}) = \beta_1 (\frac{5h_i^+}{\delta C_A} - 1) h + \beta_2 (\frac{5h_i^+}{\delta C_A}) h^2
\]

The sets of simultaneous linear equations obtained can be solved conveniently by an iterative least squares minimization technique.

RESULTS

Potentiometry—All solutions contained known amounts of HCl to lower the pH to such values that very little or no metal binding took place. The solutions were titrated with 1.0024 N NaOH. The concentrations of reactants in each sample are listed in Table I.

Proton-Diglycyl-L-histidine System—In the ligand variation, sample solutions 1, 2, and 3 were titrated. The resulting titration curves are shown in Fig. 1. These titration data were digitized and processed utilizing the PLOT program. The resulting proton liberation data \(\delta H_i^+/\delta C_A = f(pH)\) were inserted into Equation 10 at discrete steps of pH yielding a set of simultaneous equations which in turn were solved for the unknown values \(\beta_{mo}\) and \(\beta_{ma}\). Since the buffering region of the third dissociable proton was removed more than 2 pH units away from that of the next neighboring group, the data \(\delta H_i^+/\delta C_A = f(pH)\) were treated separately using a \(N \times M\) matrix where \(N = 1\) to evaluate \(\beta_{mo}\). The refined values \(\log \beta_{mo}\) of the successive protonated species \(HA, H_2A\) and \(H_3A\) are listed in Table II.

Proton Cu(ll) Diglycyl-L-histidine System—In the metal variation, solutions 4, 5, and 6 were titrated. They are shown in Fig. 2A. The titration curves were digitized and the data were processed by program PLOT to yield the intermediate data \(\delta H_i^+/\delta C_M = f(pH)\), as shown in Fig. 3. Subsequently \(pM = f(pH)\) was obtained as final output. In the ligand variation, solutions 7, 8, and 9 were titrated. The titration curves are shown in Fig. 2B. The data were digitized and subsequently processed by program PLOT to yield \(pA = f(pH)\).
TABLE II
Log stability constants (log $\beta_{pq}$) of complex species $M,H,A,$ ($M =$ Cu(II), $A =$ diglycyl-L-histidine) in 0.15 M NaCl at 25°C

<table>
<thead>
<tr>
<th>$p$</th>
<th>$q$</th>
<th>$r$</th>
<th>Log $\beta_{pq}$</th>
<th>O.D. (Log $\beta_{pq}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
<td>1</td>
<td>8.040</td>
<td>0.008</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>1</td>
<td>14.750</td>
<td>0.008</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>1</td>
<td>17.500</td>
<td>0.028</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>1</td>
<td>9.220</td>
<td>0.000</td>
</tr>
<tr>
<td>1</td>
<td>-1</td>
<td>1</td>
<td>3.648</td>
<td>0.045</td>
</tr>
<tr>
<td>1</td>
<td>-2</td>
<td>1</td>
<td>-1.991</td>
<td>0.007</td>
</tr>
</tbody>
</table>

Fig. 3. Proton displacement $\delta H_+ / \delta C_M$ as a function of pH for the Cu(II)-diglycyl-L-histidine system.

Fig. 4. Visible spectrum of Cu(II)-diglycyl-L-histidine complex (species MH$_{-2}$A).

Fig. 2. Titration curves for the system: proton, Cu(II), diglycyl-L-histidine. A (metal variation): curve 1, $C_M = 0.0$ M, $C_A = 1.715 \times 10^{-4}$ M; curve 2, $C_A = 0.828 \times 10^{-4}$ M, $C_A = 1.715 \times 10^{-4}$ M; curve 3, $C_A = 1.656 \times 10^{-4}$ M, $C_A = 1.715 \times 10^{-4}$ M. B (ligand variation): curve 1, $C_M = 0.828 \times 10^{-4}$ M, $C_A = 1.715 \times 10^{-4}$ M; curve 2, $C_M = 0.828 \times 10^{-4}$ M, $C_A = 2.572 \times 10^{-4}$ M; curve 3, $C_M = 0.828 \times 10^{-4}$ M, $C_A = 3.430 \times 10^{-4}$ M.

For the species selection of complex formation the following values for $p$, $q$, and $r$ were used: $p = 1$; $q = +5$, $-1, -2$; $r = 1, 2$. Calculation of the values $\beta_{pq}$ showed that the complex species MA, MH$_{-1}$A and MH$_{-2}$A were required to give a minimum error solution for the matrix. The refined values for the stability constants $\log \beta_{pq}$ are listed in Table II. The deprotonation of species MA and MH$_{-1}$A was very rapid over a narrow pH range (pH 4.5 to 6.0), these species never reaching significant individual concentrations. At pH 6.0 the major species was MH$_{-2}$A. Up to the limit of our titration this was the only species observed.

Spectrophotometry—A solution containing M and A in the ratio 1:2 was prepared in 0.15 m NaCl under an argon atmosphere. The metal concentration was $2.085 \times 10^{-4}$ M which corresponded to the final concentration of the complex. The absorption spectrum of the sample representing species MH$_{-2}$A was obtained at pH 8 against a blank containing peptide alone. The spectrum is presented in Fig. 4. The complex was found to have $\varepsilon_{\max} = 103$ at $\lambda_{\max} = 525$ nm.

Comparison of Binding Strength between Cu(II)-peptide and Cu(II)-albumin—It is important to compare the dissociation constant of Cu(II)-peptide with that of Cu(II)-albumin obtained under equivalent conditions and identical definition of the constant. The dissociation of Cu(II)-peptide complex and the competition of Cu(II) between albumin and peptide can be expressed as:

$$K_{Cu(II) - peptide} \quad Cu(II) + peptide$$

and

$$K_{Cu(II) - albumin + peptide} \quad Cu(II) - peptide + albumin$$

The possibility of forming albumin-Cu(II)-peptide complex was ignored because no bis complex was detected in the Cu(II)-peptide system.


The equilibrium dialysis was carried out in 0.1 M N-ethylmorpholine-HCl buffer at pH 7.63, 6° and ionic strength 0.16. Total Cu(II) concentration was kept at 3.43 × 10⁻⁸ M, while peptide concentration was varied.

### Table III

Equilibrium dialysis of ternary binding system

<table>
<thead>
<tr>
<th>[Peptide]</th>
<th>[Cu(II)]</th>
<th>[Peptide]</th>
<th>[Cu(II)]</th>
<th>[Albumin]</th>
<th>Keq</th>
<th>Kp, Cu(II)-peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻⁸ M</td>
<td>0.86</td>
<td>0.62</td>
<td>0.24</td>
<td>2.81</td>
<td>0.62</td>
<td>0.57</td>
</tr>
<tr>
<td>10⁻⁸ M</td>
<td>1.71</td>
<td>1.08</td>
<td>0.69</td>
<td>2.34</td>
<td>1.34</td>
<td>0.70</td>
</tr>
<tr>
<td>10⁻⁸ M</td>
<td>2.57</td>
<td>1.34</td>
<td>1.23</td>
<td>2.09</td>
<td>1.34</td>
<td>0.70</td>
</tr>
<tr>
<td>10⁻⁸ M</td>
<td>3.43</td>
<td>1.51</td>
<td>1.92</td>
<td>1.90</td>
<td>1.52</td>
<td>0.63</td>
</tr>
<tr>
<td>10⁻⁸ M</td>
<td>6.86</td>
<td>1.83</td>
<td>5.03</td>
<td>1.58</td>
<td>1.53</td>
<td>0.42</td>
</tr>
<tr>
<td>10⁻⁸ M</td>
<td>17.15</td>
<td>2.57</td>
<td>14.58</td>
<td>0.86</td>
<td>2.57</td>
<td>0.52</td>
</tr>
<tr>
<td>10⁻⁸ M</td>
<td>32.18</td>
<td>2.73</td>
<td>29.45</td>
<td>0.69</td>
<td>2.73</td>
<td>0.37</td>
</tr>
<tr>
<td>10⁻⁸ M</td>
<td>59.00</td>
<td>3.06</td>
<td>55.94</td>
<td>0.36</td>
<td>3.06</td>
<td>0.47</td>
</tr>
</tbody>
</table>

Therefore

\[ K_{D, \text{Cu(II)}-\text{peptide}} = \frac{[\text{Cu(II)}][\text{peptide}]}{[\text{Cu(II)}][\text{protein}]} = \frac{K_{D, \text{Cu(II)}-\text{albumin}}}{K_{eq}} \quad (13) \]

where \( K_{D, \text{Cu(II)}-\text{albumin}} \) represents the dissociation constant for Cu(II)-albumin complex which has a value of 6.61 × 10⁻⁸ (26). The \( K_{D, \text{Cu(II)}-\text{peptide}} \) values were determined from the equilibrium dialysis data utilizing ⁶⁷Cu(II) as a tracer. The results are presented in Table III. The average value of the dissociation constant for Cu(II)-peptide is 1.18 × 10⁻¹⁰.

Equilibrium Distribution of Cu(II) between Albumin, Peptide and L-Histidine—This analysis was aimed towards gaining information about the effectiveness of the designed peptide to compete for Cu(II) in presence of all the other ligands at the conditions analogous to the physiological state. There exist ternary coordination complexes L-histidine-Cu(II)-albumin (3, 26) and L-histidine-Cu(II)-peptide⁴ in the systems containing albumin, Cu(II), L-histidine and peptide, Cu(II), L-histidine, respectively. The distribution of Cu(II) among the binary and ternary complexes existing in the quaternary system excepting Cu(II)-L-histidine could be determined from equilibrium dialysis data by using ⁶⁷Cu(II) and L-[¹⁴C]histidine double labeling technique according to the similar set of equations as described previously (26). The absorbance of the solutions in the non-protein portion at 525 nm is

\[ \text{A} = \sqrt{\text{A}_{\text{Cu(II)-peptide}}} + \sqrt{\text{A}_{\text{Cu(II)-L-His}}} + \sqrt{\text{A}_{\text{L-His-Cu(II)-peptide}}} \quad (14) \]

Since Cu(II)-albumin has almost identical molar extinction as that of L-histidine-Cu(II)-albumin at this wavelength (26) and the ternary complex L-histidine-Cu(II)-peptide has the same average molar extinction value as that of Cu(II)-peptide at 525 nm, the concentration of Cu(II)-L-histidine could be obtained by utilizing the following approximation method. To the first approximation

\[ A_1 = \frac{\epsilon_{\text{Cu(II)-peptide}}}{\epsilon_{\text{Cu(II)-peptide}}} \cdot [\text{Cu(II)-peptide}]_1 + [\text{L-His-Cu(II)-peptide}]_1 \quad (15) \]

and

\[ [\text{Cu(II)-L-His}]_2 = \left[ \frac{\epsilon_{\text{Cu(II)-peptide}}}{\epsilon_{\text{Cu(II)-peptide}}} \cdot A_1 \right] - A_1 \quad (16) \]

Then

\[ A_2 = \frac{\epsilon_{\text{Cu(II)-peptide}}}{\epsilon_{\text{Cu(II)-peptide}}} \cdot [\text{Cu(II)} - \text{non-protein}] - \frac{A_1}{\epsilon_{\text{Cu(II)-peptide}}} \quad (17) \]

and

\[ [\text{Cu(II)-L-His}]_2 = \left[ \frac{\epsilon_{\text{Cu(II)-peptide}}}{\epsilon_{\text{Cu(II)-peptide}}} \cdot A_1 \right] - A_2 \quad (19) \]

The same procedures were repeated over again until the concentration of Cu(II)-L-histidine converged. The calculated data are presented in Table IV. The interpolated results of the distribution of Cu(II) binding species in the presence of equal molar concentrations of peptide and albumin and 20-fold excess of L-histidine are presented in Table V.

### Discussion

The existence of a single species in the Cu(II)-diglycyl-L-histidine system in a wide range of pH indicates the specificity of the peptide molecule for Cu(II). The peptide diglycyl-L-histidine has a total of five protons, of which three are titrable with strong base in the normal aqueous titration range. The carboxyl group, the imidazole group, and the amino group protons show buffering regions with pKₐ values of 2.72, 6.74, and 8.04, respectively. However, the two peptide nitrogens can ionize by removal of protons to form metal-peptide bonds. Since MH₂₋ₐ is the main species in this system, analysis of the proton displacement data (Fig. 3) provides important information for solving structural features of this complex. The results indicate that binding occurs at pH 4.6. The proton displacement values rise rapidly to 4 before pH 6 is reached. This means that upon metal coordination, four groups carrying protons are involved in bond formation. The carboxyl group is already deprotonated by pH 4.6. At this pH protons can come only from imidazole (pKₐ = 6.74), amino (pKₐ = 8.04), and two amide hydrogens of the peptide. It is shown clearly in Fig. 3 that upon raising the pH beyond 6, the curve starts to fall and reaches a constant value of 2 protons per Cu(II) past pH 9. Exactly 3.5 protons per Cu(II) are liberated at pH 6.74. The imidazole group at pH 9 has lost 50% of its protons. The results implicate the involvement of the imidazole group in Cu(II) binding. Similarly, at pH 8.04, which is the pKₐ of the amino group of the peptide, 2.5 protons are liberated on Cu(II) binding. At this pH 50% of the amino group are deprotonated and the results implicate further the amino group of the peptide in Cu(II) binding. Since above pH 9 a constant value of 2 protons per Cu(II)

⁴ Studies in our laboratory on the behavior of peptides in concentrated NaOH indicate that the ionization of peptide groups can be described by an H₂-acidity function. These pKₐ values are on the order of 15 (D. W. Appleton and B. Sarkar, unpublished results).
and the L-histidine concentration was about 20 tons would be provided by the amide hydrogens. No hydroxyl species were detected in the pH range 6 to 11; therefore, the protons cannot come from ionization of a water molecule. The displacement data. As shown by Hartzell and Gurd (27), 4) is also consistent with the conclusion drawn from the proton
pentapeptides lacking a histidyl residue can be characterized by a \( \lambda_{\text{max}} \) of 515 nm for a Cu(II) species involving the \( \alpha \)-amino group plus three deprotonated peptide nitrogens. A study by Bryce and Gurd (28) has shown for imidazole-containing peptides that the relative effectiveness of the three nitrogen-containing groups in lowering the \( \lambda_{\text{max}} \) values is \( \alpha \)-amino nitrogen > peptide nitrogen > imidazole nitrogen. Thus shifting of the \( \lambda_{\text{max}} \) to higher wavelength could be brought about by the replacement of an amide nitrogen with an imidazole nitrogen, i.e. (\( \alpha \)-amino) (peptide nitrogen) (imidazole nitrogen) giving a \( \lambda_{\text{max}} \) value of 525 nm noted with the species MH-\( \alpha \).

The combined evidence of the proton displacement and spectral results leaves little room for any ambiguity, and hence we can propose the structure of this species as shown in Fig. 5. The peptide diglycyl-L-histidine was designed to provide a similar geometry and array of nitrogen ligands for Cu(II) binding as have been proposed for albumin. The above results show that this peptide does mimic the Cu(II) binding site of albumin as suggested by Peters and co-workers (7-9) and Gurd and co-workers (9, 11).

Although this peptide has a similar Cu(II) binding site as that of albumin, the binding strength of Cu(II)-peptide is lower than that of Cu(II)-albumin.6 It is possible that the COOH-terminal free carboxyl group of the peptide which is absent in albumin exerts some influence on the strength with which it binds Cu(II). The presence of a \( \beta \)-carboxyl group on the NH\(_2\)-terminal aspartyl residue of albumin and the lack of it in the peptide may also contribute to this difference. It is unlikely that the conformation of the albumin molecule is responsible for the higher binding strength since the tetrapeptide from the NH\(_2\)-terminal of albumin shows the similar Cu(II) binding characteristics as those of intact molecule (11). However, Cu(II) binding strength with this fragment is not available and no definite conclusion should be entertained on this subject.

There is approximately 20 times as much total amino acids as albumin present in the human blood. However, L-histidine is the major Cu(II) binding amino acid (2, 29). In the presence of 20-fold of L-histidine over albumin in the equilibrium experiments (Table IV), the conditions could be considered analogous to the physiological state in vitro. The interpolated results (Table V) of the distribution of Cu(II) binding species in the presence of equal molar concentrations of peptide and albumin and 20-fold excess of L-histidine present some interesting findings. This tripeptide was found to compete for Cu(II). There is 17.71% of Cu(II) present in the forms of Cu(II)-albumin and

![Fig. 5. Structure of Cu(II)-diglycyl-L-histidine complex (species MIL-\( \alpha \)).](image-url)

6 Interestingly enough, the Cu(II) exchange rate from L-histidine to albumin was found to be twice as fast as that from L-histidine to peptide at pH 7.83, ionic strength 0.16 and 20°. This observation is in accord with the half binding strength of Cu(II)-peptide as compared with Cu(II)-albumin (S. Lau and B. Sarkar, unpublished results).

### Table IV

**Equilibrium dialysis data of quaternary binding system**

Conditions are the same as described in Table III. Total concentration of Cu(II)-albumin was kept constant at \( 3.42 \times 10^{-4} \) m, and the L-histidine concentration was about 20-fold that of albumin.

<table>
<thead>
<tr>
<th>[L-Histidine]</th>
<th>[Peptide]</th>
<th>[Cu(II)]</th>
<th>[Albumin]</th>
<th>[Cu(II)-L-histidine]</th>
<th>[Cu(II)-albumin]</th>
<th>[L-Histidine]</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \times 10^{-3} ) m</td>
<td>( \times 10^{-3} ) m</td>
<td>( \times 10^{-3} ) m</td>
<td>( \times 10^{-3} ) m</td>
<td>( \times 10^{-3} ) m</td>
<td>( \times 10^{-3} ) m</td>
<td>( \times 10^{-3} ) m</td>
</tr>
<tr>
<td>6.42</td>
<td>1.76</td>
<td>1.33</td>
<td>0.56</td>
<td>0.52</td>
<td>0.56</td>
<td>0.56</td>
</tr>
<tr>
<td>6.39</td>
<td>3.50</td>
<td>1.23</td>
<td>0.52</td>
<td>0.52</td>
<td>0.52</td>
<td>0.52</td>
</tr>
<tr>
<td>6.32</td>
<td>6.94</td>
<td>1.10</td>
<td>0.47</td>
<td>0.47</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>6.14</td>
<td>16.56</td>
<td>0.93</td>
<td>0.41</td>
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<td></td>
</tr>
<tr>
<td>5.86</td>
<td>32.18</td>
<td>0.62</td>
<td>0.29</td>
<td>0.29</td>
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</tr>
<tr>
<td>5.38</td>
<td>59.00</td>
<td>0.54</td>
<td>0.26</td>
<td>0.26</td>
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<td></td>
</tr>
</tbody>
</table>

**Table V**

**Interpolated data of distribution of Cu(II) binding species in presence of equimolar concentrations of Cu(II), albumin, and peptide and approximately 20-fold excess of L-histidine at pH 7.83 and 8°**

Interpolation was carried out by using equilibrium dialysis data with total L-histidine concentration in the range of 6.32 to 6.42 \( \times 10^{-4} \) m.

<table>
<thead>
<tr>
<th>Species</th>
<th>Concentration</th>
<th>Distribution of Cu(II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu(II)-albumin</td>
<td>5.22</td>
<td>15.26</td>
</tr>
<tr>
<td>L-Histidine-Cu(II)-albumin</td>
<td>0.84</td>
<td>2.45</td>
</tr>
<tr>
<td>Cu(II)-peptide</td>
<td>0.35</td>
<td>2.48</td>
</tr>
<tr>
<td>L-Histidine-Cu(II)-peptide</td>
<td>11.50</td>
<td>33.62</td>
</tr>
<tr>
<td>Cu(II)-L-histidine</td>
<td>15.80</td>
<td>46.19</td>
</tr>
</tbody>
</table>
l-histidine-Cu(II)-albumin, 36.10% in the Cu(II)-peptide and l-histidine-Cu(II) peptide forms and 46.10% as Cu(II) l-histidine. Since the peptide does not possess as high an affinity towards Cu(II) as albumin, l-histidine with a 20-fold excess molar concentration would have a much better chance to compete for the Cu(II) binding site to form the ternary complex l-histidine-Cu(II)-peptide. This could account for the much higher concentration of the ternary complex formed with peptide than that with albumin. The enhanced stability of the ternary complex l-histidine-Cu(II)-peptide rather than the binary complexes is consistent with the results of the other peptide ternary complexes studied (30).

The present findings have some important implications. A synthetic molecule, with similar specificity and affinity for Cu(II) as that of albumin, may be very useful in treating Wilson’s disease, which is a genetic defect and characterized by increased Cu(I1) deposit in the body (31). In a severe situation of hemolytic crises in Wilson’s disease, Cu(I1) must be removed very rapidly. D-Penicillamine, a strong chelator for Cu(II), has been used to treat patients (32). However, because of the lack of specificity and the limited tolerance in some patients, penicillamine does not appear to be satisfactory (33). Intravenous infusion of such a small peptide molecule or its suitable analogues and rapid clearance of the Cu(II)-complex via the kidney may be lifesaving. The preliminary results with rats indicate that an intravenous injection of this synthetic peptide is capable of removing 3 times more Cu(I1) through the urine from the treated animals than the control. This would mean that the small peptide molecule can mobilize copper in the body and help its excretion via the urine. Detailed work remains to be done. However, it appears that this approach of designing a small molecule (12, 13) to mimic certain functions of a biological macromolecule is promising and may have important biomedical applications.

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