Cupric Ion Complexes of Histidine-containing Peptides*

GRAEME P. DRYCE, ROGER W. ROESKE,† AND FRANK R. N. GURD

From the Department of Biochemistry, Indiana University School of Medicine, Indianapolis, Indiana 46207

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The binding of copper or zinc ion by sperm whale metmyoglobin appears to involve imidazole groups of histidyl residues (4, 5) that occur well removed from the NH₂ terminus (6). The binding of each cupric ion by metmyoglobin is accompanied on the average by the displacement of one or more protons beyond that may be borne by the imidazole group, presumably from peptide amide nitrogen atoms (4, 5). Such displacement of amide protons is best known for simple peptides containing a free terminal amino group (1, 7–10). Histidine-containing peptides lacking a free terminal amino group have been studied very little, except for one in which flexibility is probably quite restricted (11). For these reasons, the observations with metmyoglobin have remained unparalleled with model peptide systems (4, 12).

The present paper deals with complex formation between cupric ions and a number of histidine-containing peptides including a series of peptides of the form acetyl(glycyl),-L-histidine, acetyl(t-glycyl),L-histidiylglycine, and some of the corresponding peptides lacking the terminal acetyl group. The results show that the longer acetylated histidine-containing peptides react with cupric ions to form 1:1 complexes that dissociate protons in comparable number and in a comparable pH range to the cupric ion complexes of metmyoglobin or apomyoglobin.

EXPERIMENTAL PROCEDURE

Physical Chemical Measurements—Titration procedures, measurement of pH, and reaction with p-nitrophenylacetate were carried out as previously described (1, 13).

Peptides and copper (II) chloride were generally studied in equimolar mixtures containing enough KCl to make an ionic strength of 0.16. Standard NaOH of approximately 0.16 M was used for titration. The temperature was 25.0 ± 0.1 °.

L-Histidylglycine and glycyl-L-histidine were purchased from Cyclo Chemical Corporation. Purity was checked by thin layer chromatography and titration.

Syntheses—In the following procedures, the Rₚ values refer to thin layer chromatograms with Silica Gel G (Merck). Solvent System A is 1-butanol-acetic acid-water (4:1:1 by volume); Solvent System B is 1-propanol-water (2:1 by volume). Unless specified otherwise, the chromatograms were developed by the hypochlorite method (14). Melting points are corrected.

Acetyl-glycine p-Nitrophenyl Ester (Compound I)—Acetyl-glycine (15) (14.0 g, 0.12 mole) was dissolved in 190 ml of hot dimethylformamide and the solution was cooled quickly in an ice bath. p-Nitrophenol (16.7 g, 0.12 mole) and dicyclohexylcarbodiimide (19 g, 0.12 mole) were added, and the mixture was stirred at room temperature for 3 hours. The dicyclohexylurea was removed by filtration and the filtrate was evaporated in a vacuum. The residue, a yellow oil, was dissolved in 200 ml of ethyl acetate and washed with 0.5 M K₂CO₃-KHCO₃ buffer, pH 9.2 (6 × 50 ml). The solution was dried over Na₂SO₄ overnight and evaporated in a vacuum to an oil which was crystallized from ethyl acetate by the addition of petroleum ether. The product melted at 123–125 ° and weighed 11.2 g for a yield of 38%.

C₂₆H₃₉O₭N₇
Calculated: C 50.42, H 4.25
Found: C 50.48, H 4.34

Acetyl-glycyl-L-iminobenzylhistidine-Benzyl Ester (Compound II)—A solution of t-butoxycarbonyl glycine (1.73 g, 0.0073 mole) and p-nitrophenol (4.22 g, 0.03 mole) in 50 ml of acetonitrile was cooled to 0 ° and treated with dicyclohexylcarbodiimide (0.25 g, 0.03 mole). The mixture was stirred at 0 ° for 1 hour, then at 25 ° for 2 hours, and worked up in the same way as Compound I. After recrystallization from ether-petroleum ether, the product (6.30 g, 70%) melted at 67–69 °.

C₇₆H₇₅N₉O₭
Calculated: C 52.69, H 5.44, N 9.46
Found: C 52.57, H 5.65, N 9.59

Acetylglycyl-L-aminobenzylhistidine Benzyl Ester (Compound III)—L-Aminobenzylhistidine benzyl ester di-p-toluenesulfonate (18) (4.95 g, 0.0073 mole) was converted to its free base by dissolving it in 100 ml of CHCl₃ and stirring with 50 g of dry Amberlite IR-45 basic anion exchange resin for 30 min. The mixture was filtered and evaporated in a vacuum to a yellow syrup (quantitative yield) which was dissolved in 50 ml of CHCl₃. Acetyl-glycine p-nitrophenyl ester (1.73 g, 0.0073 mole) and a few drops of triethylamine were added and the mixture was allowed to stand at 25 ° for 3 hours. It was diluted to 250 ml with CHCl₃, washed with cold 0.1 M NaOH (3 × 30 ml), and dried over Na₂SO₄. The residue that was left after the CHCl₃ was evaporated in a vacuum was crystallized from ethyl acetate-petroleum ether. The product (81% yield) melted at 128–131 ° (decomposition) and was recrystallized from ethanol for analysis, it melted at 130–131 °.

C₆₅H₇₊N₉O₭
Calculated: C 50.42, H 4.25
Found: C 50.48, H 4.34

1 Prepared by the general method of Schwzyer, Sieber, and Kappler (16); m.p. 87–88.5 °; literature, 88.5–89 ° (17).

† Research Career Development Awardee of the United States Public Health Service.
Acetylglycyl-L-histidine (Compound IV)—A solution of Compound III (2.27 g) in 160 ml of glacial acetic acid and 40 ml of deionized water was shaken under 30 psi of hydrogen at 60° in the presence of 0.8 g of 5% Pd/C catalyst. After 8 hours, less than 5% of the iminobenzyl group remained intact, as estimated by the intensity of the spot on a chromatogram. The solution was filtered and lyophilized. The residue was dissolved in water (100 ml) and relyophilized. It was dissolved in water (8 ml) and precipitated as an oil by the addition of acetone (25 ml). The mixture was cooled in ice and centrifuged for 15 min. The supernatant was decanted and the water-acetone treatment was repeated. Finally, the suprasy residue was dissolved in water (50 ml) and lyophilized. The product, a hygroscopic solid, was chromatographically pure; \( R_f = 0.23 \) in Solvent B.

1-Butoxyxycarbonyl Glycyl-L-iminobenzylhistidine Benzyl Ester (Compound V)—This was prepared from 1-iminobenzylhistidine benzyl ester di-p-toluene sulfonate (43.64 g, 0.064 mole) and Compound II (18.0 g, 0.064 mole) according to the procedure for Compound III. The product, recrystallized from ethyl acetate-petroleum ether, melted at 101-102°. The yield was 90%.

\[
\text{Found: } C_{37}H_{23}N_5O_3, \quad \text{Calculated: } C_{37}H_{23}N_5O_3
\]

Glycyl-L-iminobenzylhistidine Benzyl Ester Dihydrochloride (Compound VI)—A solution of Compound V (1.92 g) in dioxane (10 ml) was cooled in an ice bath while anhydrous HCl was passed in until 0.6 g had been absorbed. After 15 min, the product was precipitated by the addition of ether (50 ml) and recrystallized from ethyl acetate-petroleum ether, melted at 188-190°; \( R_f = 0.15 \) in Solvent A.

\[
\text{Found: } C_{27}H_{26}Cl_2N_3O_4, \quad \text{Calculated: } C_{27}H_{26}Cl_2N_3O_4
\]

Acetylglycylglycyl-L-histidine Benzyl Ester (Compound VII)—A solution of Compound I (1.19 g, 5 mmoles) and Compound II (2.33 g, 5 mmoles) in dimethylformamide (50 ml) was treated with triethylamine (2.11 ml, 11 mmoles). After 24 hours, the solution was diluted with chloroform (250 ml) and worked up in the usual way (see Compound III). The product was recrystallized from ethyl acetate-petroleum ether; m.p. 152-154°. The yield was 75%.

\[
\text{Found: } C_{32}H_{32}N_5O_3, \quad \text{Calculated: } C_{32}H_{32}N_5O_3
\]

Acetylglycylglycylglycyl-L-histidine (Compound VIII)—Compound VII (3.05 g, 6.22 mmoles) was dissolved in glacial acetic acid (160 ml) and water (40 ml) and hydrogenated at 60°, 30 psi, for 10 hours in the presence of 5% Pd/C catalyst (1.0 g). The reaction was worked up as described for Compound IV. The white residue weighed 1.54 g (80% yield); \( R_f = 0.20 \) in Solvent B. Subjected to paper electrophoresis (2300 volts) for 60 min at pH 6.4, it showed one spot by the Pauly reaction.

\[
\text{Found: } C_{42}H_{24}BrN_4O_3, \quad \text{Calculated: } C_{42}H_{24}BrN_4O_3
\]

Acetylglycylglycyl-L-histidine Dihydrochloride (Compound IX)—Compounds VI (6.6 g, 0.0142 mole) and II (4.21 g, 0.0142 mole) were added to dimethylformamide (25 ml) and the suspension which resulted was treated with triethylamine (3.3 ml), shaken well, and allowed to stand for 16 hours. After dilution with chloroform (300 ml), the mixture was worked up in the usual way (see Compound III). The crude product was recrystallized from ethyl acetate-petroleum ether to give 6.5 g (83% yield) of Compound IX, m.p. 146-147°.

\[
\text{Calculated: } C_{35}H_{35}N_5O_6, \quad \text{Found: } C_{35}H_{35}N_5O_6
\]

Glycylglycyl-L-histidine Dihydrochloride (Compound X)—A solution of Compound IX (3.00 g, 5.47 mmoles) in glacial acetic acid (160 ml) and water (10 ml) was hydrogenated for 8 hours under the usual conditions. The product was lyophilized and suspended in dioxane (25 ml) containing anhydrous HCl (3.0 g). After 1 hour, the solution was filtered and the product was lyophilized; \( R_f = 0.27 \), Solvent B.

Acetylglycylglycylglycyl-L-iminobenzylhistidine Benzyl Ester (Compound XI)—The t-butoxycarbonyl-protecting group was removed from Compound IX by treatment with HCl in dioxane (see Compound VI). The amorphous dihydrochloride was not isolated but converted to the free base by Amberlite IR-45 in a mixture of methanol and water. A solution of the free base (2.3 g, 5.1 mmole) and Compound I (1.21 g, 5.1 mmole) in chloroform (50 ml) was allowed to stand at 25° for 24 hours. When ethyl acetate (150 ml) was added, an oil formed and slowly crystallized. The crude product was recrystallized from 95% ethanol-water to give 1.71 g (61% yield); m.p. 166-168°, \( R_f \) 0.58, Solvent B.

\[
\text{Calculated: } C_{36}H_{36}N_5O_6, \quad \text{Found: } C_{36}H_{36}N_5O_6
\]

Acetylglycylglycylglycylglycyl-L-histidine (Compound XII)—Compound XI was treated as described above for the preparation of Compound VIII. The product, a white solid, had \( R_f = 0.21 \), Solvent B.

Acetylglycylglycylglycyl-L-iminobenzylhistidine Benzyl Ester (Compound XIII)—This was prepared from Compounds II and IX by the procedure described for the synthesis of Compound XI. The product was recrystallized from ethyl acetate to give a 60% yield; m.p. 112-114°, \( R_f = 0.67 \), Solvent B.

\[
\text{Calculated: } C_{37}H_{37}N_5O_6, \quad \text{Found: } C_{37}H_{37}N_5O_6
\]

Acetylglycylglycylglycylglycyl-L-histidine Dihydrochloride (Compound XIV)—This was prepared from Compound XIII according to the procedure described above for the preparation of Compound X; \( R_f = 0.04 \), Solvent B.

\[
\text{Calculated: } C_{38}H_{38}N_5O_6, \quad \text{Found: } C_{38}H_{38}N_5O_6
\]
Acetylglycyl-L-iminobenzylhistidylglycine Benzyl Ester (Compound XVI)—A mixture of Compound I (1.00 g, 4.2 mmole), Compound XV (2.32 g, 4.2 mmole), and triethylamine (1.62 ml, 8.4 mmole) in chloroform (25 ml) was allowed to stand at 25° for 16 hours and worked up in the usual way (see Compound III). The product was recrystallized from 95% ethanol; m.p. 162-164°, 1.52 g (74% yield), RF 0.62 in Solvent B and 0.21 in Solvent A.

Calculated: C 63.53, H 5.95, N 14.25
Found: C 63.72, H 6.33, N 14.33

Acetylglycyl-L-histidylglycine (Compound XVIII)—A solution of Compound XVI (1.0 g) in 200 ml of liquid ammonia was treated with small pieces of sodium until a deep blue color persisted for 5 min. Dowex 50-WX2 on the ammonium cycle (10 g) was added, and the mixture was stirred for 30 min and allowed to evaporate. The residue was extracted with water and lyophilized to a white powder. Titration indicated that the product was the sodium salt, RF 0.21 in Solvent B.

Equilibria—The equilibria between metal ion and peptide in the following discussion can be formulated as follows.

\[
\begin{align*}
\text{Cu}^{2+} + \text{L}^{-} & \rightleftharpoons \text{CuL}^+ \quad K_1 = \frac{[\text{CuL}^+]}{[\text{Cu}^{2+}][\text{L}^{-}]} \\
\text{CuL}^+ + \text{L}^{-} & \rightleftharpoons \text{CuL}^2 \quad K_2 = \frac{[\text{CuL}^2]}{[\text{CuL}^+][\text{L}^{-}]} \\
\text{CuL}^2 + \text{H}^+ & \rightleftharpoons \text{CuL} + \text{H}_2 \text{O} \quad K_3 = \frac{[\text{CuL}]}{[\text{CuL}^2][\text{H}^+]} \\
\text{CuL} + \text{H}^+ & \rightleftharpoons \text{CuL}^+ + \text{H}_2 \text{O} \quad K_4 = \frac{[\text{CuL}^+]}{[\text{CuL}][\text{H}^+]} \\
\text{CuL} + \text{L}^{-} & \rightleftharpoons \text{CuL}_2 \quad K_5 = \frac{[\text{CuL}_2]}{[\text{CuL}][\text{L}^{-}]} \\
\text{CuL}_2 + \text{H}^+ & \rightleftharpoons \text{CuL} + \text{H}_2 \text{O} \quad K_6 = \frac{[\text{CuL}]}{[\text{CuL}_2][\text{H}^+]} \\
\text{CuL} + \text{L}^{-} + \text{H}^+ & \rightleftharpoons \text{CuL}_2 + \text{H}_2 \text{O} \quad K_7 = \frac{[\text{CuL}_2]}{[\text{CuL}][\text{L}^{-}][\text{H}^+]} \\
\text{CuL} + \text{L}^{-} + \text{H}^+ & \rightleftharpoons \text{CuL}^2 + \text{H}_2 \text{O} \quad K_8 = \frac{[\text{CuL}^2]}{[\text{CuL}][\text{L}^{-}][\text{H}^+]} \\
\text{CuL} + \text{L}^{-} + 2\text{H}^+ & \rightleftharpoons \text{CuL}_2 + 2\text{H}_2 \text{O} \quad K_9 = \frac{[\text{CuL}_2]}{[\text{CuL}][\text{L}^{-}][2\text{H}^+]} \\
\text{CuL} + \text{L}^{-} + 3\text{H}^+ & \rightleftharpoons \text{CuL}_2 + 3\text{H}_2 \text{O} \quad K_{10} = \frac{[\text{CuL}_2]}{[\text{CuL}][\text{L}^{-}][3\text{H}^+]} \\
\end{align*}
\]

Values of log $K_1$ and log $K_2$, where appropriate, were computed from the equation of Irving and Rossotti (20),

\[
\left( \frac{n}{1 - n} \right) \frac{1}{[L^-]} = \left( \frac{2 - n}{1 - n} \right) [L^-] K_1 K_2 + K_1
\]

in which $n$ and $L^-$ are defined as

\[
n = \frac{1}{[L^-]} \left( [\text{Na}^+] + [\text{H}^+] - [L^-] \right)
\]

and

\[
[L^-] = \frac{K_a}{[\text{H}^+]} \left( [L^-] - [\text{Na}^+] - [\text{H}^+] \right)
\]

RESULTS AND DISCUSSION

Syntheses—All of the peptides were prepared by the stepwise reaction of t-butoxycarbonyl amino acid p-nitrophenyl esters with amino acid or peptide benzyl esters. Removal of the t-butoxycarbonyl group was accomplished by treatment with anhydrous hydrogen chloride in dioxane. In most cases, the resulting hydrochlorides were not isolated but used directly in the next step. The benzyl ester protecting group was cleaved quantitatively by catalytic hydrogenation at room temperature for 30 min, but the iminobenzyl group of histidine required hydrogenation for 8 hours at 60° in acetic acid-water for complete removal. However, the iminobenzyl group of acetyl-glycyl-L-iminobenzyl-L-histidylglycine benzyl ester was not cleaved completely under these conditions and was finally removed by treatment with sodium in liquid ammonia (19).

TABLE I

**Formation and ionization constants for copper (II)-complexes of L-histidine-containing peptides at 25° and 0.16 ionic strength**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$pK_{aCOOH}$</th>
<th>$pK_{bimidazole}$</th>
<th>$pK_{bNE}$</th>
<th>$\log K_1$</th>
<th>$\log K_2$</th>
<th>$pK_a$</th>
<th>$pK_a'$</th>
<th>$pK_b'$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycyl-L-histidine</td>
<td>2.00</td>
<td>6.77</td>
<td>8.24</td>
<td>ND</td>
<td>ND</td>
<td>4.00</td>
<td>4.50</td>
<td>9.25</td>
</tr>
<tr>
<td>Glycerylglycyl-L-histidine</td>
<td>2.84</td>
<td>6.87</td>
<td>8.22</td>
<td>ND</td>
<td>ND</td>
<td>4.75</td>
<td>4.90</td>
<td>5.00</td>
</tr>
<tr>
<td>Acetylglycyl-L-histidine</td>
<td>3.02</td>
<td>6.85</td>
<td>8.11</td>
<td>ND</td>
<td>ND</td>
<td>6.35</td>
<td>7.10</td>
<td>8.70</td>
</tr>
<tr>
<td>Acetylglycyl-L-histidine†</td>
<td>2.99</td>
<td>7.11</td>
<td>4.13</td>
<td>3.36</td>
<td>7.30</td>
<td>8.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerylglycyl-L-histidine</td>
<td>3.08</td>
<td>7.18</td>
<td>4.24</td>
<td>3.68</td>
<td>6.50</td>
<td>7.35</td>
<td>9.25</td>
<td></td>
</tr>
<tr>
<td>Acetylglycyl-L-histidine</td>
<td>3.16</td>
<td>7.21</td>
<td>4.40</td>
<td>3.52</td>
<td>6.50</td>
<td>7.35</td>
<td>8.80</td>
<td></td>
</tr>
<tr>
<td>Acetylglycyl-L-histidine</td>
<td>3.25</td>
<td>6.86</td>
<td>3.84</td>
<td>3.68</td>
<td>6.35</td>
<td>6.90</td>
<td>11.4</td>
<td></td>
</tr>
<tr>
<td>Acetylglycyl-L-iminobenzylhistidylglycine</td>
<td>3.30</td>
<td>6.37</td>
<td>3.06</td>
<td>3.27</td>
<td>5.95</td>
<td>6.90</td>
<td>11.5</td>
<td></td>
</tr>
<tr>
<td>L-Histidylglycine</td>
<td>2.90</td>
<td>5.58</td>
<td>7.50</td>
<td>ND</td>
<td>ND</td>
<td>0.01</td>
<td>10.6</td>
<td></td>
</tr>
</tbody>
</table>

* ND, not determined.
† Peptide-copper (II) mixture (2:1).
MOLES NaOH REACTED PER MOLE Cu(II)

FIG. 1. Titration of equimolar mixture of copper (II) and glycylglycyl-L-histidine hydrochloride with sodium hydroxide. In this and all other titrations, the ionic strength was maintained at 0.16, and the temperature at 25.0 ± 0.1°C.

The results indicate that between pH 6 and 8 the form that nearly completely predominate has the copper (II) coordinated with all three of the imidazole, peptide nitrogen, and amino groups. That both imidazole and amino groups are bound is confirmed by the failure of the complex at pH 6.86 to show any activity whatever in cleaving p-nitrophenyl acetate. In the absence of the copper (II) ion at this pH, both of these groups react with p-nitrophenyl acetate (13). The copper (II) ion in this complex is almost certainly combined with 1 or 2 water molecules and may be coordinated with an oxygen atom of the carboxyl group. A crystallographic structure determination is in hand.²

Glycylglycyl-L-histidine—As an extension to the work of Martin and Edsall (21), the titration behavior of glycylglycyl-L-histidine hydrochloride in the presence of equimolar copper ions was studied. Fig. 1 shows the cooperative action of the association of the copper (II) at the α-amino group and the 1-nitrogen of the imidazole ring in the promotion of ionization of the protons on the peptide nitrogen atoms at low pH. The extensive overlapping means that the constants for this peptide quoted in Table I should be taken as approximate. The binding of the copper (II) is assumed to take place first at the imidazole group, by virtue of its lower pK value, and the liberation of protons from the α-amino group is expressed as a dissociation constant, pKα, in keeping with the terminology outlined above. Its value, together with the values of pKβ and pKβ′ (dissociation of protons from the peptide nitrogen atoms) were computed by the approximate method used in the analysis of the nickel (II)-triglycylglycine systems (23).

It is seen from Table I that the values obtained for pKα and pKβ′ for glycylglycyl-L-histidine are higher than the analogous values for glycyl-L-histidine, presumably because an initial combination of copper (II) with both the α-amino and imidazole nitrogen atoms produces a large chelate ring that does not lead directly without rearrangement into the peptide ionizations. Assignment of the last two pK values to specific peptide nitrogen atoms is difficult. Formation of a 5-membered ring from the α-amino end might be expected to yield a lower pK value than formation of a 6-membered ring from the imidazole end. However, the copper (II) will be distributed more in favor of the imidazole group by virtue of its lower pK; hence, the lower of the last two pK values (4.90) could be assigned to the peptide nitrogen adjacent to the histidine residue. The extensive overlapping of the ionizations prevented the computation of a meaningful log Kα value.

Acetylglycylglycyl-L-histidine—To study the effect of the presence of only one initial coordination locus, in addition to the carboxyl group, the behavior of acetylglycylglycyl-L-histidine was studied. The stoichiometry shown in Fig. 2 indicates that ionizations have taken place from the peptide bonds. However, the titration curve has an unusual shape when compared with that found for copper (II) triglycylglycine (10). Between the stages of 1.5 and 4 eq of NaOH, the curve can be fitted to three overlapping pKα, pKβ′, and pKβ values of 6.50, 7.35, and 9.25, respectively. The relatively high value of pKα implies that formation of a 2:1 complex, by binding of a second ligand molecule through its imidazole group, occurs. On plotting the data according to the method of Irving and Rossotti (20), this was found to be the case indeed. A linear plot was obtained in the pH region where the carboxyl pK and the pKα would not be expected to interfere (Fig. 3). The values of log K1 = 4.24 and log K2 = 3.68 are in the expected range for copper (II)-imidazole complexes (24, 25).

A knowledge of all of these constants permits the computation of the degree of formation of the intermediate complexes present in the solution at any pH, and hence the assignment of the individual visible spectra and the like. Fig. 4 illustrates the dis-

FOOTNOTE:
² H. C. Freeman, personal communication.
tribution of copper (II) in the different species of the complexes with acetylglucyclglycyl-L-histidine.

A similar pattern was obtained for the interaction of copper (II) with acetylglucyclglycyl-L-histidine. Log $K_1$ is slightly higher (Table I) in keeping with the higher affinity of the imidazole group for protons, but log $K_2$ is lower, possibly because of the mutual hindrance of the larger ligands. Values of $pK_2$ and $pK'_2$ are identical to those of acetylglucyclglycyl-L-histidine, but the $pK'_2$ of 8.80 is considerably lower than the analogous value of 9.25, presumably because of the labilizing influence of the additional glycy residue interposed between the acetyl and the nitrogen atom involved. A similar difference has been observed for the $pK'_2$ value of the complexes of triglycylglycine and tetraglycylglycine.\(^3\)

**Acetylglucycl-L-histidine**—The simplest member of the series carrying an acetyl group on the α-amino nitrogen is acetyl-L-histidine. Studies by Martin, Chamberlin, and Edsall (23) showed that the chelation phenomenon was obscured by hydroxy complex formation with subsequent precipitation. In our studies with the next higher homologue, acetylglucycl-L-histidine, the equimolar copper (II) complex appeared to give the predicted titration curve but, after two of the three required equivalents of NaOH had been added, precipitation of the complex occurred. Hence, the constants in Table I were computed from measurements on the 2:1 mixture. Again, the value of log $K_1$, 4.13, reflects the lower imidazole pK. Since the computation of log $K_2$ was carried out for a 2:1 mixture of ligand to metal, its value would tend to be more reliable than those for 1:1 mixtures. It is noteworthy that the difference between log $K_1$ and log $K_2$ is of the characteristic magnitude for the simple system of copper (II) with imidazole itself (24, 25).

The use of 1:1 copper (II)-peptide mixtures throughout the rest of this study has limited the 2:1 form to CuL$_3$ (Equation 2), for example in the case of acetylglucyclglycyl-L-histidine discussed above. Peptide dissociations from the 1:1 complex have supervened (Equations 3 to 5) and left the 2:1 form as an isolated species that only need be taken into account at low pH. In the present case of acetylglucycl-L-histidine in 2:1 mixture with copper (II), the way is more open for peptide ionizations from both ligand molecules. Two peptide ionizations are observed. Accordingly, after ionization of the two peptide protons, two alternative structures are possible for the 2:1 complex. (a) A symmetrical structure involving two imidazole groups and two peptide nitrogen atoms, one of each from each ligand peptide, and (b) a structure in which both peptide nitrogen atoms are contributed by one ligand peptide. This latter structure would correspond to the form CuL$^-$ (Equation 4) bearing a second ligand molecule coordinated through its imidazole group to the copper (II). In both bases, the 4 binding nitrogen atoms could be assigned to the four corners of a square plane, perhaps quite distorted, around the central copper (II). In either case, the higher $pK_2$ and $pK'_2$ values (7.30 and 8.05) compared with 6.50 and 7.35 for copper (II)-acetylglucyclglycyl-L-histidine can be accounted for by statistical arguments since there are two groups already bound to the copper (II) prior to the additional peptide ionizations. Or the second imidazole group could be considered as a further electron donor source mitigating somewhat the electron withdrawal by the copper (II) from the peptide nitrogen. On either basis, the $pK_2$ value would be expected to be similar to the $pK'_2$ value of the 1:1 complexes of acetylglucyclglycyl-L-histidine. The similarity in the two, 7.30 and 7.35, respectively, is striking.

It is not possible to decide unequivocally between the alternatives suggested above from titration data alone. If the fourth equivalent of NaOH represents the ionization of a second peptide proton from one ligand, then its $pK'_4$ should be about 0.8 log unit higher than $pK_2$ by analogy with the copper (II)-acetylglucyclglycyl-L-histidine system. However, on statistical grounds, the $pK'_4$ attributable to the first ionization from the peptide bond of the second ligand molecule should not be much more than 0.8 log unit higher than $pK_2$. It will be shown in a subsequent paper that optical rotatory spectra enable a decision to be made in favor of the unsymmetrical Structure b in which both peptide nitrogen atoms bound are contributed by one ligand molecule. An equilibrium between the two analogous structures

\(^3\) G. F. Bryce, unpublished observations.
has been postulated for the 2:1 complex of copper (II) and triglycylglycine (7).

Glycylglycylglycyl-L-histidine—To study the distribution of copper (II) on a peptide containing two coordination sites and a total of five potentially strong chelation loci, the equimolar mixture of copper (II) and glycylglycylglycyl-L-histidine hydrochloride was investigated. The sixth potential ligand group, the carboxyl, is presumably much less effective. Fig. 5 shows the results obtained. The first two equivalents of NaOH consumed probably account for the protons on the α-amino group and the imidazole side chain of the ligand hydrochloride. The break in the curve shows that this process is separated from the subsequent ionizations, whose $pK_a$ values are 6.35, 7.10, and 8.80. These are very similar to the values obtained for the acetylated derivative (Table I) and very different from the values found for glycylglycyl-L-histidine, indicating that cooperation between the α-amino and imidazole groups is almost absent. This finding accords with the expected effect of the size of the potential initial chelate ring.

The absence of additional dissociations below about pH 5.5 suggests that the structure at this stage consists of either a large chelate ring or else a polymeric structure in which copper (II) ions bridge ligand molecules through α-amino and imidazole groups. Otherwise one would expect certain elements of a complex of the type of copper (II)-diglycylglycine to be present, and hence a $pK_a$ value of about 5.10 (10).

Only 4 nitrogen atoms can occupy the corners of the square plane around the copper (II), a plane established by the 3 co-ordinated peptide nitrogen atoms. The next problem is to decide whether, in addition to the 3 peptide nitrogen atoms, the α-amino group or the imidazole N1 lies at the fourth corner. The possibility of forming three 5-membered rings in the final complex would seem to argue for the former alternative. However, the analogous $pK_a$ values for copper (II)-triglycylglycine are 5.45, 6.90, and 9.25 (10), which are not very similar to those found here. Moreover, the striking similarity to the $pK_a$ values of copper (II)-acetylglycylglycylglycyl-L-histidine, coupled with the advantage for the imidazole group of its lower $pK_a$, weigh against the structure involving the imidazole and three peptide nitrogen atoms. Evidence will be presented in a later paper to confirm the latter configuration on the basis of visible spectra and optical rotatory dispersion measurements.

Acetylglycyl-α-histidylglycine—Since the aim of this study is to establish an accurate model for the interaction of copper (II) with a segment of a polypeptide chain, the next logical step is to consider the effect of copper (II) on peptides where histidine is not COOH-terminal. Two such peptides are acetylglycyl-α-histidylglycine and the derivative carrying a benzyl group on Nα of the imidazole.

The comparison of values in Table I shows that substitution of the negatively charged carboxylate in the histidyl residue increases the acidity of the tertiary imidazole nitrogen and lowers the $pK_a$ to 6.86; the additional benzyl group further lowers it to 6.37. Table I shows that this is reflected in a substantially lower value for the association constants with copper (II). The log $K_1$ values form a consistent pattern with other members of the series and a good correlation with the $pK_a$ values of the imidazole groups is illustrated in Fig. 6.

A point of interest is the finding that log $K_2$ is higher than log $K_1$ for the complex of copper (II) and acetylglycyl-L-iminobenzylhistidylglycine which is possibly due to some van der Waals attraction between the two benzyl groups overcoming the inherent difficulty of binding the second ligand. Compare, for example, the quite small change between log $K_1$ and log $K_2$ in the system copper (II)-carbobenzoxy-L-prolyl-L-histidylglycaminamide (11).

The introduction of the benzyl group also causes a lower value of $pK_a$ (Table I). This effect will be discussed below.

It should be emphasized here that the building of molecular models indicates that the presence of the benzyl group on Nα of the imidazole ring is very unlikely to affect the course of the subsequent ionizations from the complex. Substitution on N1 of the imidazole group would interfere drastically with complex formation (26).
Analysis of the titration curve above pH 10, making appropriate corrections for activity coefficient and free hydroxyl ion concentration, revealed a further ionization with a pK value of 11.4 for the free peptide and 11.5 for the benzyl derivative. The magnitude suggests the assignment to ionization of a coordinated water molecule, similar to that found with copper (II)-diglycylglycine (10). It should be noted that the benzylimidazole group could not release a proton from its second nitrogen atom.

Another chelation site is present in the above complexes, namely the peptide bond between the histidyl and COOH-terminal glycine residues. Since the only locus of initial attack is the imidazole group, chelation to this peptide nitrogen atom involves the unlikely formation of a 7-membered ring. Reactions of this type have been more or less ruled out by the work on the copper (II) complexes of carbobenzoxy-L-prolyl-L-histidylglycaminamide (11).

L-Histidylglycine—In context here is the behavior of L-histidylglycine in which the peptide bond is on the COOH-terminal side of the histidyl residue (Fig. 7). Titration curves of the equimolar mixture of dipolar peptide and copper (II) can be explained as follows. Coordination is assumed to occur initially at the imidazole group with subsequent release of protons from the α-amino group on adding the first equivalent of NaOH. The ionization constant for this displacement was not computed. The second equivalent is almost surely the ionization of the proton from the peptide bond, represented as pKₐ in Table I. Now these 3 nitrogen atoms cannot lie in the same plane within bonding distance to the copper (II). The peptide may be considered as a derivative of glycylglycine, and with this in mind a structure can be visualized in which two of the four corners of the square plane are occupied by the α-amino and peptide nitrogens. Slight distortion in the direction of a flattened tetrahedral configuration may permit the imidazole nitrogen to approach within bonding distance of the copper (II) by assuming an apical position with reference to the postulated square plane.

Assignment of pK Values in High pH Range—An important question arising from the above discussion is the assignment of pK values in the high pH range, 9 to 12. It was suggested by Martin and Edsall (21) that the pKₐ value of 9.25 in the equimolar complex of glycyl-L-histidine and copper (II) or nickel (II) be assigned to ionization of not of a water molecule, but of the proton from the pyrrole nitrogen of the imidazole ring. This represents a substantial lowering, in the presence of the metal ion, from a postulated value of 14.2 (27). Any pKₐ of greater than 9.0 must be examined as to its origin. The pKₐ values for coordinated water molecules vary from 9.37 for glyoxyl glycine to 11.9 for diglycylglycine (10) and are therefore dependent on the number of ionized peptide bonds in the ligand in question. The measurement of the rate of hydrolysis of p-nitrophenyl acetate by copper (II) complexes of diglycylglycine (10) ruled out the possibility that the pKₐ value of 6.90 could be ionization of a coordinated water molecule, similar to that found with copper (II)-diglycylglycine (10). Therefore, the presence of two ionized peptide bonds raises the pK of a bound water molecule ionization to values in excess of 11. Hence, the pKₐ values of 9.25, 8.50, and 8.70 for the copper (II) complexes of acetylglucylglycyl-L-histidine, acetylglucylglycylglycyl-L-histidine, and glycylglucylglycylglycyl-L-histidine must either be ionization from the third peptide bond or the pyrrole nitrogen. Considering the pKₐ values of 11.4 and 11.5 for acetylglucylglycyl-L-histidylglycine and its benzyl derivative it is seen that, unless they are fortuitously similar, neither can represent anything but a water molecule ionization since no pyrrole proton is present on the latter peptide. By analogy, therefore, the pyrrole ionization would be the less favored explanation for the other complexes discussed above.

The pKₐ of 10.6 obtained for L-histidylglycine falls within the range for the water ionizations considered above. If it is rather high for a simple dipeptide complex (28), the explanation may well lie in the peculiar symmetry suggested above for it.

Systematic Relations between Equilibrium Constants—It has already been shown (Fig. 6) that log Kₑ values for the formation of copper (II) complexes of acetylated peptides are linearly related to the pK values for the imidazole group. This can also be expressed as a dependence of pKₑ on log Kₑ. It is to be expected that the stabilizing effect of the copper (II) on the peptide proton will be strongly influenced by the extent of electron donation by the group acting as the initial coordination locus. This is probably the major factor involved since the environment of the peptide bond in question is very similar in the examples considered. Hence, a higher pK value for the imidazole group will be associated with a greater degree of electron donation to the copper (II) which will be reflected in a higher value for pKₑ and conversely.

Included in Fig. 8 is a point for glycylglucylglycyl-L-histidine which, as noted previously, behaves in a similar manner to the acetylated derivative with respect to the pKₑ value.

The broken curve in Fig. 8 represents values of the imidazole pKₑ corrected to allow for the difference between an unsubstituted imidazole group and the N-substituted benzyl derivative. On the assumption that neither nitrogen atom in the imidazole ring normally bears more than three substituents, it follows that the substituted and unsubstituted peptides should be compared after
plexes of the two series of peptides. It will be taken up together in a separate paper.

The relative amounts of chelate stabilization in the initial complexes of copper (II) ions per molecule (4), net displacement will be much smaller than if the \( \Delta p_H \) values were referred to a previously denatured protein. Electrostatic effects, although small, should cause some net loss of protons from the copper (II)-reacted protein at constant pH, perhaps amounting to 0.1 or 0.2 of an equivalent per mole (5). Furthermore, the set of imidazole groups that enter into hydrogen ion equilibrium may well be changed by the reaction of the protein with copper (II) (4). The measurements of \( p \)-nitrophenyl acetate cleavage reported previously (4) imply no great change in the activity of basic imidazole groups at constant pH, from which it follows that the number of protonated imidazole groups is nearly the same in the copper (II)-reacted unless the factors mentioned above do indeed alter the apparent \( p \)K values considerably or modify the catatysis in some undetected way.

Table II lists for comparison the values for the average numbers of hydrogen ions displaced from the peptide bonds in the 1:1 complexes of copper (II) with acetylglycylglycyl-L-histidine, acetylglycylglycyl-L-histidine, acetylglycylglycyl-L-histidylglycine, and acetylglycyl-L-iminobenzylhistidylglycine. These values are computed from the titration studies, taking as a starting point the anionic form of the peptide in which the imidazole group is unprotonated. The closest parallels between the peptide and protein complexes are observed at pH 7.50 and above, and the deviations are not very large up to pH 11. A strict parallel in behavior is not to be anticipated because of the

making the statistical correction of 0.30 log unit. This quantity has been subtracted from the value for the substituted peptide as a matter of convenience. A similar correction has been omitted from Fig. 6 where it should be equally valid. This inconsistency is intentional because, as pointed out below, the plot in Fig. 6 may have considerable practical application under circumstances where the uncorrected values would naturally be used.

It appears that the relation shown in Fig. 6 can be extended beyond the present series of peptides. The peptide carboxy-L-prolyl-L-histidylglycinamide has a \( p \)K for the imidazole group of 6.42 and \( \log K_1 \) of 3.28 (11). On the basis of this \( p \)K the curve in Fig. 6 leads to \( \log K_1 \) of 3.16. This peptide is remarkable in that a peptide ionization does not occur, possibly because of steric hindrance.

**Comparison with Titration Behavior of Complexes of Metmyoglobin and of Apomyoglobin**—The copper (II) complexes of metmyoglobin and apomyoglobin have been extensively investigated by hydrogen ion titration (4, 5). The most convenient basis for comparison with the model peptides is a consideration of the number of protons liberated on binding a copper (II) ion \( (\Delta p_H/\Delta p_M) \) as a function of \( \Delta p_H/\Delta p_M \). Table II lists a set of values of \( (\Delta p_H/\Delta p_M) \) for metmyoglobin and apomyoglobin obtained from the difference in the number of protons bound in the absence and presence of about 4 bound copper (II) ions at each of a series of \( \Delta p_H/\Delta p_M \) values. The values in Table II rise from 1.3 to above 3.0 between pH 6.50 and 11.00. The expression \( (\Delta p_H/\Delta p_M) \) is roughly equivalent to the number of hydrogen ions ionized from peptide bonds, especially at pH 7.50 and above where the contribution of imidazole-bound hydrogen ions is probably quite negligible (4, 5, 29).

Below pH 7.50, the value of \( (\Delta p_H/\Delta p_M) \) will include any contribution arising from the net displacement of hydrogen ions from imidazole groups. Because the native structure contains imidazole groups masked in the basic form, groups that are probably exposed by reaction with several copper (II) ions per molecule (4), net displacement will be much smaller than if the \( \Delta p_H \) values were referred to a previously denatured protein. Electrostatic effects, although small, should cause some net loss of protons from the copper (II)-reacted protein at constant pH, perhaps amounting to 0.1 or 0.2 of an equivalent per mole (5).

Furthermore, the set of imidazole groups that enter into hydrogen ion equilibrium may well be changed by the reaction of the protein with copper (II) (4). The measurements of \( p \)-nitrophenyl acetate cleavage reported previously (4) imply no great change in the activity of basic imidazole groups at constant pH, from which it follows that the number of protonated imidazole groups is nearly the same in the copper (II)-reacted unless the factors mentioned above do indeed alter the apparent \( p \)K values considerably or modify the catatysis in some undetected way.

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- **Table II**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Value of ( (\Delta p_H/\Delta p_M) ) at pH</th>
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<tr>
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<td>6.50</td>
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<tr>
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<td>Acetylglycyl-L-iminobenzylhistidylglycine</td>
<td>1.00</td>
</tr>
</tbody>
</table>

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4 The extension to simple dipeptides bears on the question of the relative amounts of chelate stabilization in the initial complexes of the two series of peptides. It will be taken up together with optical rotatory evidence for the histidine peptides in a separate paper.
different environments of the peptide bonds in the protein complexes. How important these may be is indicated by the small but systematic differences in $(-\Delta^{pK}/p_M)$ for metmyoglobin and apomyoglobin (5), observed even under conditions where the native structures are definitely altered (4, 5, 30)\(^2\).

Side-chain interactions could favor or hinder the apposition of the peptide nitrogen atoms to the copper (II). One could expect that the results for the binding of 4 metal ions per molecule (4, 5) could be biased one way or the other at different stages in the complex formation.

It is useful to test the possibilities for closer agreement with the results for the protein complexes by estimating the values of $pK_v$, $pK_v'$, and $pK_v''$ that follow from the relations in Table I and Figs. 6 and 8. For example, the intrinsic $pK$ for the imidazole groups in myoglobin has been estimated at 6.7 (29, 30). Hence, from Fig. 8, the $pK_v$ in a corresponding copper (II) complex of the kind reported here should be of the order of 6.2. If, furthermore, the spacing among $pK_v$, $pK_v'$, and $pK_v''$ for acetylglycylglycylglycyl-$L$-histidine (Table I) is taken to apply in the present hypothetical case, then the latter two values will be about 7.0 and 8.5, respectively. With such a set of values, $(-\Delta^{pK}/p_M)$ is computed to be 0.92 and 1.39 at pH 6.50 and 7.00, respectively.

The agreement with values for metmyoglobin and apomyoglobin is relatively good, and it is clear that most of the discrepancy at these pH values can be removed by some reasonable choices of $pK_v$ values. The general adequacy of peptides of this type as models for the metmyoglobin and apomyoglobin interactions with copper (II) is further supported by measurement of optical rotatory properties to be reported separately.

Finally, two points should be stressed to indicate the limitations of these model peptides as models for the proteins considered. The first is that the relation in Fig. 6 predicts what is probably too low a value of log $K_1$ at least for the first few copper (II) ions bound per myoglobin molecule. By taking the $pK$ of the imidazole groups in the protein as 6.7, a value of log $K_1$ of 4.0 for the first few copper (II) ions bound is predicted from Fig. 6. Even allowing for subsequent ionization steps to stabilize the complexes, it appears probable from the available binding data (4, 12) that log $K_1$ should be greater than 4.0 for the first copper (II) ions bound. This limitation is difficult to evaluate critically because the $pK$ value of 6.7 must be a composite of individual values with an unknown spread. By the stage of the fourth or fifth copper (II) ion bound the value of 3.6 for log $K_1$ can scarcely be judged too low (4).

One extremely strong site of binding is to be expected. Two histidine residues, EF4 and EF5, are adjacent in the sequence (6). It is planned to study model peptides containing adjacent histidine residues set in sequences corresponding to the EF segment of the myoglobin (4, 12). From the studies of the complexes of histidylhistidine that have been reported (21, 33), however, it seems obvious that strong chelation should be expected through both imidazole groups of the adjacent residues, at least the one intervening peptide bond, and probably a second peptide bond. In this case two histidyl residues would provide only the one copper (II)-binding site.

The second limitation to be considered is that a naive appeal to these model peptides would lead one to expect that all 12 histidyl residues in myoglobin would be expected to act as loci of quite strong binding sites. However, strong binding levels off at 6 to 7 metal ions bound (4, 5). Presumably the explanation is to be found in limitations on the folding of the peptide chain around the copper (II) ions. These limitations may arise from inevitable steric hindrance of bulky side chains, stabilization of residual secondary structure, even in the disrupted protein (23-35), or mutual interference between chelation patterns of histidine residues that occur close to each other in the myoglobin sequence.

It is planned to extend this work as previously outlined (4, 12) to a variety of peptides corresponding in their sequences to histidine-containing segments of the myoglobin sequence.

**SUMMARY**

1. The following peptides have been synthesized and their interaction with copper (II) ions studied: acetylglycyl-$L$-histidine, acetylglycylglycyl-$L$-histidine, acetylglycylglycylglycyl-$L$-histidine, glycylglycyl-$L$-histidine, glycylglycylglycyl-$L$-histidine, acetylglycyl-$L$-iminobenzyl-$L$-histidineglycine, and acetylglycyl-$L$-histidylglycine. The t-butoxycarbonyl amino acid-$p$-nitrophenyl esters were reacted in the stepwise synthesis with the amino acid or peptide benzyl esters. Protective groups were removed by standard methods. Commercially available glycyl-$L$-histidine and $L$-histidylglycine were also studied.

2. The peptides were titrated in equimolar mixtures with copper (II) with sodium hydroxide. The titration results were generally interpreted in terms of first and second association constants for the combination of copper (II) with a first and, to a lesser extent, a second peptide molecule, $K_1$ and $K_2$, followed by ionizations of hydrogen ions from the complexes, $K_n$, $K_n'$, and $K_n''$. The latter ionizations were attributed to peptide nitrogen atoms.  

3. Acetylglycyl-$L$-histidine was studied in 2:1 proportion to copper (II). A complex was formed that involved two imidazole groups and 2 peptide nitrogen atoms as ligands to the copper (II). 

4. The greatest attention was paid to the acetylated peptides as possible models for copper (II)-binding structures in proteins. After initial combination with the imidazole group and possible chelation to a first peptide bond, the copper (II) combines firmly to the peptide nitrogen atom with ejection of a hydrogen ion and the formation of a stable chelate. A second and third peptide bond were able to react in the same manner to form at least a quadridentate chelate. For example, with acetylglycylglycylglycyl-$L$-histidine, $pK_v$, $pK_v'$, and $pK_v''$ were 6.50, 7.35, and 8.80, respectively.

5. Evidence against a second hydrogen ion dissociation from the imidazole ring in the multidentate copper (II)-peptide complexes was obtained, mainly by finding that the complex of the iminobenzyl derivative showed a dissociation with $pK_v''$ of 11.4, indistinguishable from that for the corresponding compound with an unsubstituted histidyl residue.

6. The peptide bond on the COOH-terminal side of the histidyl residue in the two appropriate tetrapeptides studied did not appear to be involved in the complex formation with copper (II) to the extent of hydrogen ion dissociation. A probable difference in stereochemistry of the glycyl-$L$-histidine and $L$-histidylglycine complexes may be correlated with these observations.

7. Linear relations were found in the acetylated peptide series between the $pK$ for hydrogen ion dissociation from the imidazole group and (a) log $K_1$; and (b) $pK_v$.\(^2\)

\(^2\) No attempt is made here to discuss the interaction of a single copper (II) ion with the unaltered structure (4, 31, 32).
8. A rough parallel between the course of hydrogen ion dissociation as a function of pH for the acetylated peptides studied and for metmyoglobin and apomyoglobin was shown. Reasonable assumptions can be employed to refine the agreement above pH 7.5. Possible sources of deviation at lower pH are discussed. Peptides of this sort are concluded to be quite good models for the interaction of sperm whale myoglobin with copper (II) ions.

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Cupric Ion Complexes of Histidine-containing Peptides
Graeme F. Bryce, Roger W. Roeske and Frank R. N. Gurd