Optical Rotatory Properties of Cupric Ion Complexes of Simple Dipeptides*

GRAEME F. BRYCE, J. M. H. PINKERTON, L. K. STEINRAUF, AND FRANK R. N. GURD

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

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Titrations and spectral measurements of sperm whale metmyoglobin (2) and apomyoglobin (3) in the presence of cupric ion indicate that the metal ion may form chelates with imidazole groups and some other proton-bearing groups such as peptide amides. The titration and spectral properties of complexes of certain histidine-containing peptides have proved to be similar to those of the protein (4). Another technique that has proved effective in establishing parallels between complexes formed by the protein and by model peptides is measurement of optical rotatory dispersion. The formation of successive copper-peptide complexes may be observed by a combination of measurements of hydrogen ion titration, optical absorption spectra, and optical rotatory dispersion spectra. The optical rotatory dispersion spectra may be expected to be particularly sensitive to chelate formation. First, the constraint exerted by the metal ion on the peptide ligand, and any other perturbing effects of the metal ion, would be expected to modify the dispersion curve characteristic of the peptide. This might be observed as a shift in the wave length of the first Cotton effect in the ultraviolet region or by changes in the values at positive and negative extrema. Second, it may be expected that the d-d transitions of the cupric ion may be rendered detectably optically active if an asymmetrical ligand is bound as a bidentate or higher chelate (5, 6). This type of effect may be observed in the visible region where the free ligand shows rather small optical activity. The results obtained with histidine-containing peptides will follow. The present report deals with the optical rotatory properties of a series of simple peptides containing no side-chain groups capable of forming coordinate bonds with cupric ion. As an adjunct to these studies and in order to form a firmer base for interpreting the equilibria with larger peptides, two separate computation methods have been programmed for determining equilibrium constants in these systems.

EXPERIMENTAL PROCEDURE

Unless noted otherwise all of the peptides were supplied by Cyclo Chemical Corporation. The L-leucylglycine was obtained from Hoffmann-La Roche and Company. Peptides were stored over drying agent at -20°.

Titrations—Titrations were performed at 25° as previously described (7, 8). The calomel electrode was thermostatted separately and connected by a bridge containing saturated KCl (9). Reference buffers were phthalate (pH 4.01), equimolar phosphate (pH 6.86), and borax (pH 9.18) prepared according to directions issued by the National Bureau of Standards. The Radiometer pH meter 4 was used. Cupric chloride was mixed with an equimolar concentration, usually 0.01 M or 0.005 M, of peptide in the dipolar ionic form, and KCl stock solution was added to 0.16 M. The titrations were made stepwise with increments of standard NaOH. At extremes of pH, activity coefficient corrections were made from experimental measurements. The purity of the peptides was checked by titration in the absence of added copper as well as by chromatography in 1-mm layers of Silica Gel G (Merck) with butanol-acetic acid-water (4:1:1 by volume).

Absorption Spectra—In most cases, solutions were prepared containing the same concentrations of cupric ion and peptide as for the titrations and with integral molar proportions of NaOH added. Spectra were scanned with the Cary model 14 spectrophotometer. Results are reported in terms of molar absorbance, ε.

Optical Rotatory Dispersion Measurements—The Bendix polarimetric instrument was used as described previously (1). Usually the same cell and solution were used as for the absorption measurements. The results are expressed in terms of molecular rotation, [M] = (α) × (M/100), where (α) = (observed rotation in degrees per dm), d is expressed in decimeters, c in grams per ml, and M is the molecular weight. A light path of 10 mm was used most often, but for some purposes 1 mm was chosen.

RESULTS

Titrations—All of the dipeptides studied showed the characteristic titrations of 1:1 mixtures of cupric ion with dipeptides having no ligand atoms in the side chains (7, 10-15). The neutralization of the first two equivalents of alkali by equimolar ratios of copper (II) ion and dipeptide corresponds to the removal of hydrogen ion from the ammonium group and the amide bond. A third equivalent is involved in practice if the hydrochloride of the peptide is used. The equilibria involved are

\[ \text{Cu}^{2+} + L^- \rightleftharpoons \text{CuL}^+ \quad K_1 = \frac{[\text{CuL}^+]}{[\text{Cu}^{2+}][L^-]} \quad (1) \]

\[ \text{CuL}^+ \rightleftharpoons \text{CuL} + \text{H}^+ \quad K_c = \frac{[\text{CuL}][\text{H}^+]}{[\text{CuL}^+]}, \]

where \( L^- \) denotes the anionic form of the ligand peptide (e.g., glycyglycinate), \( \text{CuL}^+ \) a first complex formed directly, and \( \text{CuL} \)
a complex derived by the further displacement of a proton from the amide linkage. A further stage to yield the complex CuL− corresponds to the loss of a proton from a coordinated water molecule. The complex CuL− is fully defined at the equivalence point. If less than the equivalent amount of alkali is added, a type of dimer corresponding to the combination of CuL− with CuL is formed (7, 14).

The species CuL and CuL− may be obtained nearly pure by adjusting the proportion of alkali added. The species CuL+ cannot usually be obtained without considerable proportions of L− and CuL. The estimation of the proportion of CuL+ present depends critically on the values of K1 and Kc chosen.

Computations—The values of log K1 and pKc were computed by the methods of Datta and Rabin (11) and of Koltun, Fried, and Gurd (7) programmed in FORTRAN for the IBM 709 and IBM 1410 computers. In the first method, an experimentally derived parameter, ϕ, was plotted against the reciprocal of hydrogen ion concentration and extrapolation, by the method of least squares, of the linear portion of the curve yielded an initial value of log K1. Substitution of this value into an expression containing known quantities and Kc yielded a set of solutions for K<sub>1</sub> or K<sub>c</sub> was recycled, increased or decreased by 1%, until the minimum mean variance in Kc was obtained, at which point the constants best fitted the data. The following restrictions were imposed: (a) the chosen solutions of the quadratic for K<sub>c</sub> had to be positive and lie within the range of hydrogen ion concentrations used, and (b) mean variances in pK<sub>c</sub> were only accepted as valid when the number of data points selected was constant for different values of log K1. This number was at least half of the number of original data points and always greater than 15. Selected hand calculations agreed well with the above method.

The second method involved an initial estimate of pK<sub>c</sub>, and a test for consistency in the computed values of log K1 over the pH range studied. Recycling of the initial pK<sub>c</sub> value in 0.1 unit increments from 1 unit below to 1 above produced a series of straight lines when the resultant values of log K1 were plotted against pH. The slopes of these lines were plotted against the inserted value of pK<sub>c</sub>. The best values of log K1 and pK<sub>c</sub> were thus obtained when the slope was zero.

A comparison of these two methods and a hand calculation by the second method is shown in Table I. The spread of the results by the different methods is approximately 0.1 log unit. The titration values for the glycylglycine system obtained in this study gave much more self-consistent computations than the values used by Koltun, Fried, and Gurd (7). Different weighting in the latter values must account for the values obtained of 4.96 and 3.90 for log K1 and pK<sub>c</sub>, respectively (7). The values for glycylglycine in Table I compare quite well with some of the most carefully obtained published values, although close comparison cannot be made because of differences in ionic strength and temperature (10, 11, 14).

For the computations in Table I, the following pK<sub>c</sub> values were determined for the dissociations of the amino and carboxyl groups, respectively, of the free peptides: glycyl-L-valine, 8.20 and 3.26; glycyglycine, 8.16 and 3.23; glycy-L-alanine, 8.24 and 3.22; L-alanylglycine, 8.15 and 3.23.

**Optical Rotatory Dispersion of Free Ligands**—The optical rotatory dispersion curves of L-alanine at three different pH values are shown in Fig. 1. The pH values were chosen to convert the amino acid nearly quantitatively into the cationic, dipolar ionic, and anionic forms, respectively. In each case, the long wave length transition in the ultraviolet shows a positive Cotton effect with a positive extremum on the long wave length side of 220 mμ. A more powerful negative Cotton effect appears to superimpose on the negative limb of the first. Urry and Eyring summarize the evidence that the first Cotton effect is due to an n-π* transition in the acyl moiety, and relate in these terms L-alanine, L-lactic acid, and L-histidine (16).

Fig. 1 shows that the rotation at the positive extremum near 220 mμ is affected considerably by the state of ionization of the molecule. The Cotton effect is much stronger for the cationic form than for the dipolar and anionic forms. The lower wave lengths were not explored since they could not be studied in the presence of copper ion.

Fig. 2 shows the curves for two dipeptides, in which the L-
alanyl residue is the only asymmetrical moiety, L-alanylglycine and glycyl-L-alanine. The curves for the two peptides, Fig. 2, a and b, show generally opposite trends. L-Alanylglycine, like L-alanine, has positive Cotton effects for the different forms. In all of the cases with these two peptides, the best developed long wave length Cotton effects are observed when the alanyl residue itself is charged.

Fig. 3 shows curves for L-alanyl-D-alanine and D-alanyl-L-alanine. Observed rotations are somewhat greater, in the opposite sense, for the latter peptide, but generally less than for the peptides shown in Fig. 2. These observations may reflect some internal compensation but may also reflect shifts in the wave lengths of the transitions determining the two incompletely resolved Cotton effects.

Fig. 4 shows the curves for the different ionic forms of L-alanyl-L-alanine. The long wave length Cotton effect is negative. The cationic form shows an early reversal, like the same form of glycyl-L-alanine (Fig. 2b). If on this evidence, one may consider the second, carboxyl-terminal alanyl residue to be the dominant contributor to the observed dispersion pattern, it may be that it is because its methyl group is near both chromophoric acyl groups, the amide and carboxyl. The results for all of these dipeptides share with those for amino acids such as alanine, histidine (16), and others (17, 18) that the long wave length Cotton effect has its extremum close to 220 mμ or below.

Comparison of the curves for L-alanylglycine (Fig. 2a) with those for L-alanine (Fig. 1) indicates that the extrema for the dipolar and probably the cationic forms are more positive in the case of the peptide. Likewise, there is an indication in Fig. 4 that at least the anionic form of L-alanyl-L-alanine reaches a very high negative rotation that might compare well on an absolute basis with the free amino acid, even after account is taken of the two residues represented by the dipeptide. A definite evaluation of relative rotatory strengths is not an immediate concern.

**Optical Rotatory Dispersion of Cupric Complexes**—In Fig. 5, the dipolar form of L-alanine is compared with the bis-alanato forms of zinc (II) and copper (II). The curve for the zinc complex has the same general shape as that for the free amino acid. The curve for the copper (II) complex is quite different. The Cotton effect in the ultraviolet is negative, with the extremum at about 270 mμ, and a crossover to positive values near 245
Fig. 4. Optical rotatory dispersion curves for L-alanyl-L-alanine. The solid curve represents the anionic form, $L^-$, in 0.5 M NaOH. The dashed curve represents the cationic form, $L^+$, in 0.6 M HCl. The dash-dot curve represents the dipolar form, $L^*$, pH 5.8, ionic strength 0.16.  

The chromophore in the case of the bis-alanato copper (II) complex may be identified with an absorption peak with a molar absorbance of 6900 at 235 mp similar to that seen in the corresponding glycine complexes (8). By analogy with the glycine complexes, the structures of the copper (II) and zinc (II) complexes of L-alanine may be expected to be quite similar except for tetragonal distortion in the former (19, 20). The optically active transition at the longer wave length observed with bis-L-alanato copper (II) may represent a perturbation of a carboxyl transition or a charge transfer process.

Fig. 5 also shows an interesting region of positive rotation for bis-L-alanato copper (II) in the visible range up to the limit of the instrument near 625 mp. The curve indicates that one or more optically active transitions are associated with the d-d transitions of the copper (II). The absorption maximum in the visible is at 620 mp for which $\varepsilon_{\text{max}}$ is 57. Such observations with copper (II) complexes of L-amino acids have been known for some time (21).

It was found that a mixture rich in the 1:1 complex, L-alanato copper (II), showed a generally similar shape to that of the bis complex, except that the rotation, even in the visible region, was negative. A negative extremum was not reached within the limits of manageable absorbance.

The characteristics of the optical rotatory dispersion curves of the copper (II)-dipeptide complexes are similar to those of the bis-L-alanato copper (II) in showing optically active transitions in the ultraviolet between 250 and 350 mp and again in the visible region. The regions of the curves dominated by each transition are now sufficiently close together that fusion of Cotton effects occurs as a rule.

The curves for 1:1 complexes of copper (II) and L-alanyl-glycine are shown in Fig. 6. Curves 1, 2, 3, and 4 represent mixtures to which 0, 1, 2, and 3 eq of NaOH have been added. Curve 1 represents very little complex formation and mainly indicates the sign of the long wave length ultraviolet Cotton effect for the free peptide. Curve 2 represents formally the species Cu$L^+$, but as pointed out above contains some Cu$L$ as well as free peptide. It shows clearly changes in the visible region as well as a positive extremum near 315 mp. Curve 3 corresponds to Cu$L$. The positive extrema at 555 mp and 320 mp are well

![Graph showing optical rotatory dispersion curves for L-alanine compared with the bis-L-alanato complexes of zinc (II) and copper (II). The dot-dash curve represents the dipolar form of alanine, $L^*$; the dashed curve represents bis-L-alanato zinc (II); the solid curve represents bis-L-alanato copper (II), shown on an expanded scale for the portion above 400 mp. For the latter two curves, the molar rotation is expressed per mole of the complex.](http://www.jbc.org/)
defined. Curve 4 corresponds to CuL. The positive extrema are shifted to 530 μm and below 310 μm.

Fig. 7 shows similar curves for 1:1 mixtures of copper (II) with glycy1-L-alanine and glycy1-D-alanine. As might be expected, the results for the two peptides mirror each other almost exactly. All of the complexes show at least one Cotton effect in the ultraviolet region between 290 and 330 μm. In the visible region, the clearest extrema are shown by the CuL- complexes in which one coordinated water molecule is dissociated to the hydroxide form.

Fig. 8 shows the curves for 1:1 mixtures of copper (II) with glycy1-L-valine and L-valylglycine. The curves for the former (Fig. 8a) are quite similar to those for the glycy1-L-alanine complexes (Fig. 7a). Conversely, those for L-valylglycine (Fig. 8b) are quite similar to those for the L-alanylglycine complexes (Fig. 6) if one allows for a levorotatory dominance at the low wave length end of the spectrum studied. Curves for L-leucylglycine complexes (not shown) are quite similar to those in Fig. 8b.

An example of the curves obtained when both residues of the dipeptide are derived from optically active amino acids is shown in Fig. 9 for 1:1 copper (II) complexes of L-alanyl-L-alanine.

The optical rotatory dispersion of copper (II) complexes of d-alanyl-L-alanine and L-alanyl-D-alanine was studied but is not shown. These complexes show much less optical activity. The Cotton effect or effects in the visible are barely detectable. Complexes of d-alanyl-L-alanine show a trend to positive rotations down to 280 μm, and those of L-alanyl-d-alanine show a slightly less pronounced trend to negative rotations. Considerable internal compensation seems to be evident.

Absorption Spectra—The characteristics of the visible absorption spectra of several of the copper (II)-dipeptide complexes of the forms CuL and CuL- are shown in Table II. As has been observed previously, the conversion of CuL to CuL- did not affect the absorption spectra very much (7, 10, 14). The present extinction values for L-valylglycine complexes may be somewhat low, but the other complexes fall in with previous experience where comparisons can be made (7). It has been reported pre-
FIG. 9. Optical rotatory dispersion curves for 1:1 complexes of copper (II) and L-alanyl-L-alanine. Curves 1, 2, 3, and 4 represent mixtures to which 0, 1, 2, and 3 eq, respectively, of NaOH have been added.

Table II
Absorption characteristics of some copper (II)-dipeptide complexes

The results for 630 to 640 nm and for 870 nm represent values at absorbance peaks. Those at 520 to 530 nm represent shoulders for which the values are estimated with some uncertainty.

<table>
<thead>
<tr>
<th>Dipeptide</th>
<th>CuL</th>
<th>CuL⁻</th>
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<tbody>
<tr>
<td></td>
<td>λ max</td>
<td>ε max</td>
</tr>
<tr>
<td>L-Alanylglycine</td>
<td>530</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>635</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>870</td>
<td>36</td>
</tr>
<tr>
<td>Glycyl-L-alanine</td>
<td>555</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>635</td>
<td>93</td>
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<tr>
<td></td>
<td>870</td>
<td>33</td>
</tr>
<tr>
<td>Glycyl-L-valine</td>
<td>530</td>
<td>26</td>
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<tr>
<td></td>
<td>870</td>
<td>96</td>
</tr>
<tr>
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<td>26</td>
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<tr>
<td></td>
<td>630</td>
<td>83</td>
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<td></td>
<td>870</td>
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</table>

Previously that complexes of this sort show an inflection near 230 nm with an associated extinction of about 4000 m⁻¹ cm⁻¹ (8).

Discussion
Simple amino acids are recognized to form chelate complexes with copper (II). The formation constants are distinctly larger than for the ammonia complexes (22). In the crystal structure of bis-glycinato copper (II) monohydrate, the two ligands are in a cis arrangement, whereas there is evidence that in the dihydrate they are trans (19). The 2:1 complexes of simple amino acids with copper (II) in solution may form mixtures of both cis and trans complexes. This possibility may limit the value of quantitative comparisons of the optical rotatory properties of such amino acid complexes with dipeptide complexes.

In the bis-glycinato copper (II) monohydrate, the 2 amino nitrogen atoms and 1 oxygen atom from each carboxyl group are coordinated with copper to form an approximately planar square about the copper atom. A fifth coordination is filled by oxygen of a water molecule at a somewhat greater distance (2.41 Å as opposed to 1.93 to 1.99 Å). At a greater distance still, 2.74 Å, a sixth coordination is occupied by a carboxyl oxygen of another glycine residue separately bonded to another copper atom. In solution, this last coordination is probably replaced by a loosely bound water oxygen atom. Generally, similar structures have been found for the corresponding complexes containing α-amino-α-butyreric acid (23) and proline (24).

Of the three dipeptide complex structures, CuL⁺, CuL, and CuL⁻, that of CuL has been confirmed for the crystalline state by x-ray diffraction analysis of copper (II) monoglycylglycine trihydrate (25). The copper is 5-coordinated. Four ligand atoms are approximately in one plane, the α-amino nitrogen, amide nitrogen, a carboxyl oxygen, and a water oxygen. The copper lies just above the plane, and at a relatively large distance above it in an apical position lies a second water oxygen. The bond lengths and bond angles bring out characteristics that are found also in copper complexes of larger peptides (26-28). The most prominent of these are that the bond angles around copper (II) within the chelate rings are less than 90°, and that the copper-nitrogen bond to the amide nitrogen is much shorter than to the terminal amino group (1.87 Å compared with 2.03 Å). The latter bond and the copper-oxygen bonds, with the exception of the long bond to the apical water oxygen, are comparable to those found in other complexes (24, 26). The stoichiometry of this structure is well established in solution (7, 10-15), and the structure is well supported by infrared measurements in D₂O (14).

The structure of the first complex, CuL⁺, is less firmly established. The cupric ion, once coordinated to the α-amino group, is able to coordinate either with the amide oxygen (12) or with the amide nitrogen (14). In either case, a five-membered ring is formed. The value of log K₁ for the formation of CuL⁺ is about 5.5 (Table I). This is somewhat higher than the log K₁ of 4.4 for the first complex with ammonia (30), a fact that implies definite chelate formation. However, it is much less than the value of log K₁ of about 8.5 for the formation of the first glycine complex (31). These observations are in keeping with Structure I suggested by Kim and Martell (14), in which the copper ion is coordinated principally with the α-amino group,
the protonated amide nitrogen, the terminal carboxyl group, and a water molecule. This structure is compatible with careful infrared studies in D₂O (14). The structure in which the copper (II) is coordinated with the amide oxygen (12) cannot be completely ruled out. On the one hand, it is difficult to obtain CuL⁺ in high concentration relative to L⁻ and CuL, as mentioned previously, so that positive evidence of the kind offered by Kim and Martell is scarcely overwhelming. On the other hand the structure in which the amide oxygen is coordinated is sterically allowed, and indeed is observed in the crystal structure of glycylglycylglycine copper (II) chloride sesquihydrate (26, 27). The 4-fold bond of the amide nitrogen atom in Structure I seems also to be permissible, to judge by the crystal structure of sodium glycylglycylglycine copper (II) monohydrate (26). Structure I does not take account of 1 or 2 water molecules probably also coordinated with the copper (II).

The form CuL⁻ for a dipeptide has not been studied crystallographically. Marked changes in absorption spectrum are not seen (7, 14). Presumably 1 of the 2 coordinated water molecules in the structure CuL dissociates a hydrogen ion. This type of compound has two interesting reactions. One is the reaction with CuL to form a kind of dimer (7, 14). Kim and Martell have suggested a reasonable structure (14), especially in the light of the crystalline structure of sodium glycylglycylglycine copper (II) hydrate (24). The second reaction is the catalysis of the cleavage of p-nitrophenyl acetate (7). The formation of the hydroxyl complex may enhance the distortion of the coordination about the central atom.

The results in Figs. 6 to 9 indicate that the hydroxy complexes, CuL⁻, most consistently show the strongest Cotton effects in the visible and ultraviolet ranges. The full interpretation of the visible range is hampered by the limitations of the instrument. However, the CuL⁻ form of the copper (II)-glycyl-L-alanine system (Fig. 7a) has been studied by measurements of circular dichroism with the Roussel-Jouan Dichrographe kindly made available by Dr. T. S. Piper. In the visible range two negative bands were observed, one centered about 520 mp and the other somewhat above 600 mp and incompletely developed at the limit of the instrument. These bands correspond to bands in the absorption spectrum of the solution, appearing as a shoulder at 520 mp and a peak at 630 mp (Table II). The curve in Fig. 7a may be interpreted in these terms, at least for the 520-mp transition. A dextrorotatory displacement clearly affects the whole curve in this region. It seems clear that the first Cotton effect in the ultraviolet in this case is positive, although the positive extremum of the Cotton effect with cupric ion show first extrema near 220 mp and 310 to 320 mp. These complexes show an absorbance maximum at 630 to 640 mp and a shoulder at 520 to 530 mp. It appears that both of these wave length regions are associated with optical active transitions. Optically active transitions in this wave length range are observed best in the complexes with the strongest chelate bonds and strongest ligand fields about the metal.

Computer procedures for calculating equilibrium constants in these systems are described.

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