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The Complex Formation of Copper(II) with GHL* and Related Peptides

M. J. A. RAINER and B. M. RODE

Institute of Inorganic and Analytical Chemistry, University of Innsbruck, A-6020 Innsbruck, Innrain 52a, Innsbruck, Austria

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Abstract

The formation constants for complexes of Cu(II) with GHL and a series of related dipeptides were determined by means of potentiometric titration and ESR spectroscopy in aqueous solution. The complex formation of the related peptides AH, LH, HL, GL and VL is compared to that of GHL. The somewhat higher affinity of GHL to Cu(II) as compared to AH and LH seems to be a poor explanation for the biological functions of GHL. A dimeric Cu(II)–HL complex is detected, which displays an ESR spectrum at room temperature. The ESR spectra of the different complexes and the influences of structures on the spectra are discussed.

Introduction

The tripeptide GHL is co-isolated with copper and iron from human plasma at about 200 ng/cm^3 [1, 2]. GHL alters the rate of growth and survival of cultured hepatoma cells and hepatocytes [2]. The enhancement of growth by GHL is increased when the transition metals copper, iron and zinc are added in combination with GHL, whereas the addition of these metals without GHL decreases the number of cultured cells [3]. This fact led to the conclusion that GHL should bind the toxic metals in a utilizable form [3]. Pickart and Thaler [3] postulated a mechanism for the copper transport in plasma wherein GHL should act as a 'low-weight-carrier' and transfer copper to HSA. This mechanism is supported by our work on the Cu(II)–GHL–HSA system [4].

However, there are some controversial points of view about the specificity of GHL and the structure of its Cu(II) complexes in comparison to related peptides which display no or only low biological activities [5]. In order to find out the specific properties of GHL in complex formation and to elucidate its manifold biological functions [6], in the present work the complex formation of GHL with Cu(II) is compared to that with related dipeptides. The system Cu(II)–GHL has been investigated so far by potentiometric titration [4, 7, 8], NMR spectroscopy [9, 10], ESR spectroscopy [4, 10, 11], optical absorption spectroscopy [4, 7, 11] and crystallographic structure determination [12]. Among the other Cu(II)–dipeptide systems only the system Cu(II)–AH has been investigated by potentiometric titration [13].

Experimental

Materials

$CuCl_2 \cdot H_2O$ (analytical grade) was dried at 130 °C to constant weight. All peptides were obtained from Serva and were used without further purification. Aqueous solutions were prepared using CO_2-free distilled water; the ionic strength was 0.1 M $NaNO_3$ in H_2O. Acid and base were Titrisol products (Merck).

Potentiometric Titrations

Titrations were carried out at various metal/ligand ratios ranging from 1:1 to 1:4. The Cu(II) concentration was 0.001–0.0025 M. The systems were titrated with 0.4 M NaOH at 37 °C using a 0.2 cm^3 burette (Gilmont) after adding an appropriate amount of HCl.

ESR Experiments

About 7–15 samples of 0.1 ml per titration were taken from the titration vessel by a micropipette and were immediately frozen in liquid nitrogen.

The concentration of Cu(II) was 0.0023–0.0025 M; the titration volume was 3–5 cm^3. After defrosting the samples, the spectra were recorded at 37 °C.

*Abbreviations used throughout: AH L-histidine-N^2-L-alanyl; ESR electron spin resonance; GG glycine-glycyl; GH histidine-N^2-glycyl; GHL L-lysine-N^2-(-N-glycyl-L-histidyl); GL L-lysine-N^2-glycyl; HA alanine-histidyl; HG glycine-histidyl; HL L-lysine-N^2-histidyl; HV valin-histidyl; HSA human serum albumin; LH L-histidine-N^2-L-leucyl; NMR nuclear magnetic resonance; VL L-lysine-N^2-L-valyl.

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Apparatus

For the pH measurements a Schott pH-meter CG 803 and an Ingold electrode calibrated with standard buffer solutions (Merck) were used.

The ESR spectra were recorded on a Varian E 104 spectrometer (calibrated microwave frequency = 9.097 GHz) in tubes with only 1 mm diameter (Wilmad Cat. Nr. 800) in order to reduce the dielectric losses caused by water as solvent.

Calculations

All calculations were carried out at the CDC 74 computer of the University of Innsbruck. 20–30 points per titration and 400 points per ESR spectrum (digitized with a Summagraphics ID 2000) were included.

Methods

The principle of both methods has been described earlier [4, 14].

Results

All formation constants are of the form:

$$pK_{pqr} = -\lg\left(\frac{M_pH_qL_r}{m^p h^q l^r}\right)$$

from the reaction:

$$pM + qH + rL \rightleftarrows M_pH_qL_r$$

(M, H, L = Cu(II), proton, ligand in its anionic form; p, q, r = stoichiometric numbers for M, H, L; m, h, l = concentration of free Cu(II), proton, free L)

Table I gives the pK_{pqr} values obtained by both methods. Almost all constants pK_{pqr}^{tit} and pK_{pqr}^{ESR} are identical within their standard deviations.

TABLE I.

p	q	r	GHL[a]				
			pK_{pqr}^{tit} [b]	dpK_{pqr}^{tit} [c]	pK_{pqr}^{ESR}	dpK_{pqr}^{ESR} [d]	literature
0	4	1	−26.51	0.19	–	–	−27.05[e]
0	3	1	−23.99	0.09	–	–	−23.25[e]
0	2	1	−17.66	0.10	–	–	−17.47[e]
0	1	1	−10.01	0.20	–	–	−10.09[e]
1	1	1	−19.00	0.68	n.d.	n.d.	
1	0	1	−16.12	0.10	−16.28	0.18	−14.83[e]
1	−1	1	−7.01	0.40	−6.94	0.25	−5.87[e]
1	−2	1	3.01	0.31	3.01	0.72	4.50[e]
1	1	2	−29.02	0.90	−29.02	0.69	−27.38[e]
			AH				
0	3	1	−16.81	0.13	–	–	−17.40[f]
0	2	1	−14.29	0.05	–	–	−14.76[f]
0	1	1	−7.79	0.07	–	–	−8.05[f]
1	1	1	n.d.	n.d.	−12.08	–	−12.38[f]
1	0	1	−8.93	0.10	−8.76	0.24	−8.92[f]
1	−1	1	−4.58	0.06	−4.76	0.10	−4.81[f]
1	−2	1	4.85	0.17	4.79	0.19	4.36[f]
1	0	2	−14.66	1.10	−14.93	0.32	−15.61[f]
1	−1	2	n.d.	n.d.	−7.42	0.65	−7.88[f]
			LH				
0	3	1	−16.52	0.15	–	–	
0	2	1	−14.16	0.06	–	–	
0	1	1	−7.72	0.06	–	–	
1	1	1	n.d.	n.d.	−11.82	0.10	
1	0	1	−8.98	0.10	−9.01	0.18	
1	−1	1	−4.63	0.08	−4.66	0.11	

(continued on facing page)

TABLE I. *(continued)*

p	q	r	pK_{pqr}^{tit} [b]	dpK_{pqr}^{tit} [c]	pK_{pqr}^{ESR}	dpK_{pqr}^{ESR} [d]
1	−2	1	4.79	0.16	4.71	0.18
1	0	2	−14.32	0.84	−14.86	0.32
1	−1	2	−7.18	0.86	−7.55	0.35
			GL			
0	3	1	−20.54	0.24	–	–
0	2	1	−17.78	0.12	–	–
0	1	1	−10.01	0.15	–	–
1	1	1	−15.58	0.32	−15.36	0.65
1	0	1	−11.60	0.05	−11.52	0.14
1	−1	1	−2.76	0.13	−2.63	0.13
1	−2	1	7.33	0.12	7.40	0.24
1	1	2	−25.01	0.28	−24.55	0.39
1	0	2	−15.72	0.63	−16.12	0.20
1	−1	2	−6.03	0.43	−6.05	0.24
			VL			
0	3	1	−20.56	0.16	–	–
0	2	1	−17.47	0.05	–	–
0	1	1	−10.00	0.07	–	–
1	1	1	−14.83	1.30	−15.18	0.47
1	0	1	−11.68	0.07	−11.82	0.19
1	−1	1	−2.76	0.21	−3.10	0.22
1	−2	1	7.24	0.19	7.03	0.52
1	1	2	−24.10	1.06	−24.57	0.35
1	0	2	−15.35	1.05	−15.85	0.36
1	−1	2	−5.77	1.10	−5.89	0.79
			HL			
0	4	1	−25.05	0.29	–	–
0	3	1	−23.03	0.08	–	–
0	2	1	−17.38	0.07	–	–
0	1	1	−10.09	0.09	–	–
1	2	1	−21.56	0.83	−21.30	–
1	1	1	−18.60	0.11	−19.05	0.35
1	0	1	n.d.[g]	n.d.[g]	n.d.[g]	n.d.[g]
2	0	2	−27.80	0.35	−28.50	0.70
1	−1	1	−3.11	0.27	−3.12	0.61
1	−2	1	7.38	0.47	7.24	0.60
1	2	2	−34.88	0.21	−35.43	0.37
1	1	2	−26.30	0.31	−26.23	0.51
1	−1	2	−6.94	0.35	−7.10	0.62

[a] for pK_{pqr} values of GHL see also ref. 4; [b] for pK_{0q1} values and further references see also ref. 15; [c] if pK_{pqr}^{tit} is varied by $\pm dpK_{pqr}^{tit}$ experimental error (Σ_i (base$_{added}^{calcul}$ − base$_{added}^{exper}$)^2) is doubled; [d] if pK_{pqr}^{ESR} is varied by $\pm dpK_{pqr}^{ESR}$ experimental error (see ref. 4, 14) is doubled; [e] 37 °C, 0.15 M NaCl; see ref. 8; [f] 25 °C, 0.2 M KNO_3; see ref. 13; [g] contribution too low to calculate pK_{101}; [n.d] not detected by this method.

Figure 1 illustrates one example of a measured and simulated spectrum and the contribution of the spectra of all species present in solution, whereas Figs. 2, 3 and 4 show three distributions of species (for GHL see ref. 4) as a function of pH.

Discussion

As pointed out in our previous communications [4, 15], one cannot compare the affinity of ligands to the metal ion by only comparing the pK_{pqr}, if

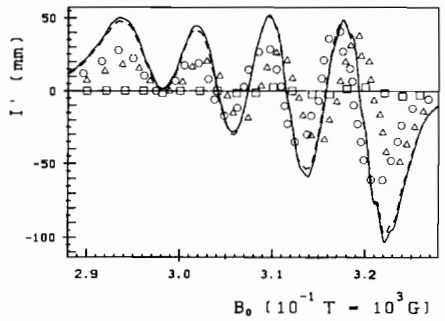

Fig. 1. Experimental (———) and calculated (– – –) spectra and the contribution of the spectra of the pure species. Concentration of copper(II) 0.00245 M. Concentration of AH 0.00257 M. pH = 9.61 (○ $M_1H_{-1}L_1$; △ $M_1H_{-2}L_1$; □ $M_1H_1L_2$).

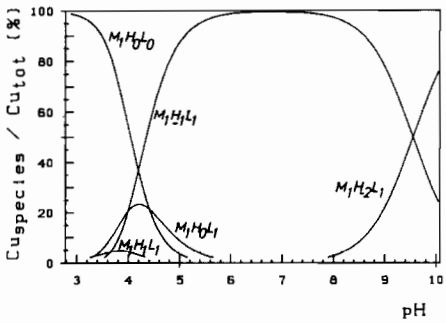

Fig. 2. Distribution of species as a function of pH for Cu(II)–AH. Concentration of copper(II) 0.0025 M. Concentration of AH 0.0025 M.

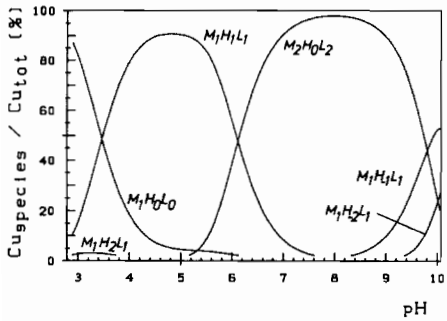

Fig. 3. Distribution of species as a function of pH for Cu(II)–HL. Concentration of copper(II) 0.0025 M. Concentration of HL 0.0025 M.

their protonation behaviour is different. For instance, Lau and Sarkar [7, 10] have drawn such a conclusion from the comparison of pK_{101} for GH and GHL (for details see ref. 4) and they explain the much lower pK_{101} for GH in relation to GHL by the involvement of an additional functional group in the latter complex.

Figure 5 illustrates that GHL indeed has the highest affinity, but also that the difference between

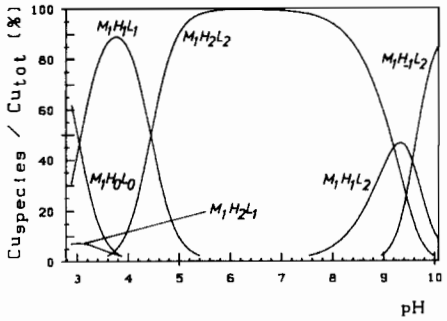

Fig. 4. Distribution of species as a function of pH for Cu(II)–HL. Concentration of copper(II) 0.0025 M. Concentration of HL 0.0100 M.

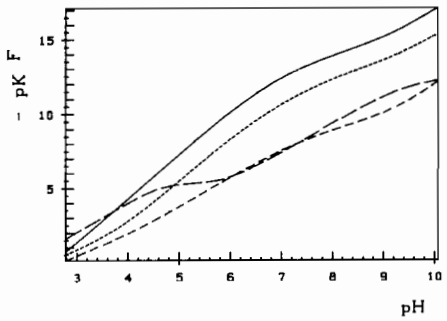

Fig. 5. pK^F as a function of pH for the complex formation of copper(II) with different ligands. Copper(II): ligand = 1:1. ——— ligand = GHL, ------ ligand = AH, – – – ligand = GL, — — — ligand = HL, pK_{pqr} were taken from Table I; pK_{0qr} are pK_{0qr}^{tit}; pK_{pqr} (p ≥ 1) are pK_{pqr}^{ESR}.

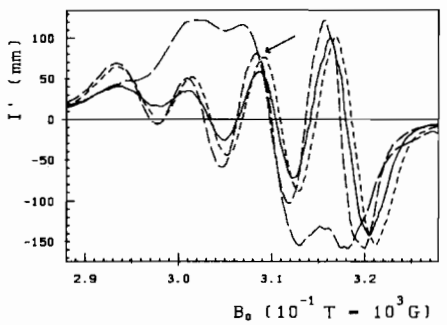

Fig. 6. Spectra of the pure species. ——— GHL: $M_1H_0L_1$, ------ AH: $M_1H_{-1}L_1$, – – – GL: $M_1H_0L_1$, — — — HL: $M_2H_0L_2$; this calculated spectrum contains some percent of the monomer $M_1H_0L_1$ (see text and Fig. 9). ⟶ see Fig. 9. LH displays nearly the same spectra as AH with a slight shift to higher B_0 and higher A values. VL displays nearly the same spectra as GL with a slight shift to higher B_0 and higher A values.

its pK^F and the pK^F of AH is only about 1.7 orders of magnitude at physiological pH.

Our results demonstrate that there is no qualitative difference in the complex formation of Cu(II) with

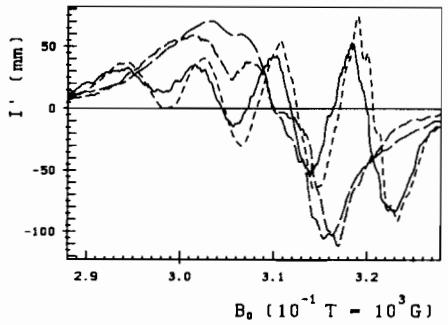

Fig. 7. Spectra of the pure species. ——— GHL: $M_1H_{-1}L_1$, ------ AH: $M_1H_{-2}L_1$, – – – GL: $M_1H_{-1}L_1$, — — — HL: $M_1H_{-1}L_1$.

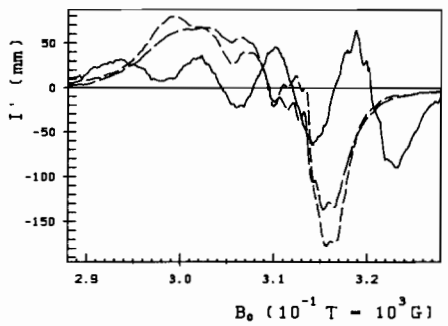

Fig. 8. Spectra of the pure species. ——— GHL: $M_1H_{-2}L_1$, – – – GL: $M_1H_{-2}L_1$, — — — HL: $M_1H_{-2}L_1$.

GHL and AH, LH and GH respectively. Figures 6–8 show the spectra for some of the species which are predominant in the range of pH 5 to 10 in 1:1 solutions of M and L.

One can see from Fig. 6 that the predominant species in the neutral pH range display nearly the same shape with the exception of HL; this shape is also very similar to that of aliphatic dipeptides [14] and can be explained by the presence of the 'spectra determining structure element', the Cu(II)–amide-nitrogen bonding [16].

If these complexes lose a proton, the picture changes completely. Very similar spectra are obtained for GL, VL and HL and another very similar group for GHL, AH and LH (Fig. 7). The first group displays similar spectra as the corresponding complex of GG and other aliphatic dipeptides [14]; at this deprotonation step the change in the spectra is most probably due to the deprotonation of one water molecule bound in the complex.

The difference in the behaviour of the latter group as compared to the first may be due to the loss of the proton from imidazole (as suggested by Martin and Edsall [17] for GH), instead of the detachment of a proton from water as proposed in our previous communication [4]. The pK value for imidazole as an acid was determined to be about 14.2 [18]. GHL, GL, VL and HL can dissociate a further proton, but the loss of this proton does not influence the shape of the spectra to a great extent, so that we assume that it is detached from the ammonium group of lysine [4], which is uncomplexed in all cases. The lysine residue in GHL, GL, VL and HL therefore plays a minor role in the stabilisation of the complexes and is limited to an embedding effect in GHL–Cu(II) complexes [4].

An interesting result is the strange shape of the spectra of 1:1 mixtures of Cu(II) and HL in the neutral range (see Fig. 6), although there is no doubt that the 'spectra determining structure element' (the Cu(II)–amide-nitrogen bond) is present at this pH. This kind of shape has never been found in our previous studies [4, 14, 16, 19]. Further investigations showed that this complex must be a dimer (as reported for the very related dipeptides HG, HA and HV [13, 20]. There are some reasons for this suggestion:

(1) The simulation of the titration curves is slightly improved if the dimer is taken into account.

(2) 1:1 mixtures of Cu(II) and HG display a very similar spectrum in the neutral range; the shape is also related to that of the dimeric complex of carnosine with Cu(II) [21].

(3) Following the method of Brown *et al.* [21] we recorded some spectra at different temperatures (Fig. 9), observing an isosbestic point, so that the

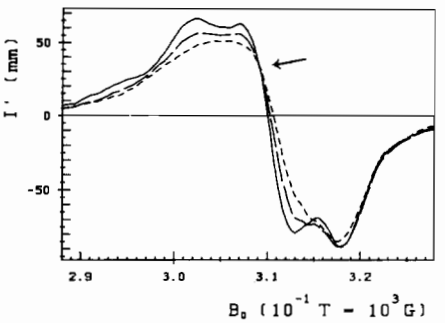

Fig. 9. Influence of the temperature on the spectrum of a 1:1 solution of Cu(II)–HL at pH = 7.6 (0.0025 M). ------ 8 °C, — — — 20 °C, ——— 42 °C, ⟶ isosbestic point. For $M_1H_0L_1$ we predict a very similar spectrum for HL and GL. Assuming this the isosbestic point can be explained from Fig. 6 (⟶).

actual spectrum should contain two spectra of different species. This observation is the same as reported for Cu(II)–carnosine solutions [21].

By increasing the temperature the contribution of the monomer ($M_1H_0L_1$) is slightly increased. Despite this the contribution of the monomer must be below 10% because the monomer is supposed to have a spectrum like the monomer of $M_1H_0L_1$ for GL in Fig. 6, with very high intensities.

The same effect of increasing contribution of the monomer is observed if we use more dilute 1:1 solutions (0.0005 M is the limit of the spectrometer). Unfortunately we could not record spectra of considerably higher concentrated 1:1 solutions (where the dimer should be the only complex in the solution), because of the limited solubility of the dimer, but the shoulders nearly disappeared in 1:1 solutions with a concentration of 30 mM.

(4) The double integral of the spectrum of the dimer is about two times higher than for the monomer spectra.

(5) It is known and supported by our investigations [4, 15] that all three important binding sites (imidazole-, N-termina-amino- and amide-nitrogen) can interact with one metal ion in GHL [12], LH, AH and GH [22] as well. Because of steric reasons this is not possible in HG, HA, HV [13] and HL. The imidazole nitrogen remains free or can bind the second Cu(II).

In such a structure the two Cu(II) ions are distant enough to prevent spin compensation.

Conclusion

It seems evident that the biological activity of GHL does not originate from the somewhat higher affinity to Cu(II) as compared to related peptides (*e.g.* GH, AH, LH). The only explanation is the presence of the uncomplexed ϵ-ammonium group in the $M_1H_0L_1$ complex acting as a receptor site. This is in good agreement with the findings of May *et al.* [8], who recently presented a simulation of the low-molecular-weight-equilibria in the biofluid including GHL.

The second interesting finding is that whenever histidine is the second amino acid (counted from the amino end of the peptide), histidine may be able to act as an acid and lose the normally non-titrable pyrrolic proton upon complex formation.

From the spectroscopic point of view, the observation of the spectrum for the dimer is most interesting because up to now there have been very few examples of dimeric Cu(II) complexes which display an ESR spectrum in solution at room temperature [21, 23].

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