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Melanostatin and its Complexes with Cu(II) and Ni(II) Ions. Spectroscopic and Potentiometric Studies

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The complexes formed between Melanostatin (MIF), L-prolyl-L-leucyl-glycinamide, and H^+, Cu(II) and Ni(II) have been studied spectroscopically and potentiometrically. MIF forms a mono-hydrogen complex (log K = 8.80) through protonation of the prolyl nitrogen. With Cu(II) it forms a series of 1:1 complexes as the pH is raised by the successive ionization of amide protons. The final complex containing four nitrogen donor atoms in a square planar arrangement around the Cu(II) is pink in colour and forms above pH 8, becoming the major species by pH 9. With Ni(II) only the final square planar complex is identifiable.

Introduction

Melanostatin (MIF), L-prolyl-L-leucyl-glycinamide, is a hypothalamic hormone and a therapeutic agent for Parkinson's disease [1]. It is also an interesting chelating agent since it has both proline and leucine residues in its sequence, as well as a potential amide donor group on a peptide C-terminal.

The proline residue in a peptide sequence [2], is not involved in direct coordination to a metal ion unless it is in the N-terminal position [2–4]. However, it can influence the symmetry around the metal ion as well as ligand conformation [2].

These features of MIF as a ligand prompted us to study the Cu(II) and Ni(II) ion interactions with this molecule both by potentiometric and spectroscopic methods. The results of these studies are presented in this communication.

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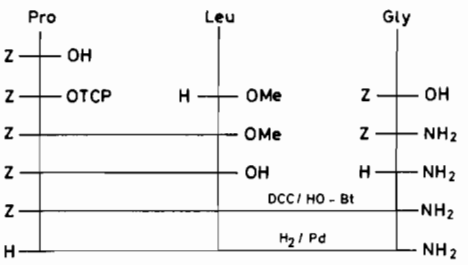
Experimental

The synthesis of L-prolyl-L-leucyl-glycinamide (Pro-Leu-Gly-NH_2, MIF) was carried out by coupling of Z-Pro-Leu-OH [5] with Gly-NH_2 according to the following scheme:



The final product was characterized by m.p. 120–123 °C, $[\alpha]_D^{20} = -48.8°$ (c2, ethanol) (m.p. 120–122 °C in ref. [5]). Elemental analysis for N: 19.85% calc., 20.14% exp. ($C_{13}H_{24}N_4O_3$). IR spectra: 3400–3220 cm^{-1} (NH), 1680 cm^{-1} (amide I), 1560 cm^{-1} (CO, amide II).

Spectrophotometric studies

$Ni(ClO_4)_2 \cdot 6H_2O$ and $Cu(ClO_4)_2 \cdot 6H_2O$ (Fluka) were used as the metal ion source. Solutions with molar ratios of metal:peptide of 1:5 and 1:1 for the Ni(II) and Cu(II) systems respectively were used. The copper(II) concentration was equal to 0.005 *M*, while the nickel(II) concentration was 0.002 *M*.

Absorption spectra were measured on a Beckman UV 5240 spectrophotometer. Circular dichroism (CD) spectra were recorded on an automatic recording spectropolarimeter JASCO-J-20. All CD results are expressed in terms of $\Delta\epsilon(\epsilon_l - \epsilon_r)$. Electron paramagnetic resonance (EPR) spectra were taken on a JEOL JES-ME-3X spectrometer at liquid nitrogen temperature and at 9.12 GHz (X-band).

Potentiometric Studies

Complex formation constants were calculated from potentiometric titration curves carried out at 22 °C using total volumes of 1.5 or 2.0 ml. Alkali was added using a 0.1 ml micrometer syringe and pH changes were followed using a microcombined electrode (Russell pH) calibrated in terms of hydrogen ion concentrations. All titrations were carried out in solutions of ionic strength 0.10 *M* (K[NO_3]). Ligand concentration was 0.002 *M* (approx.) and metal: ligand ratios studied were 1:1 and 1:2 with Cu^{2+} and 1:2 and 1:5 with Ni^{2+}. Calculations were made with the aid of the MINIQUAD computer program [6].

Results and Discussion

Spectroscopic Studies

Cu(II)–MIF solutions. The plot of the pH dependence of the d–d transition energy in Cu(II)–MIF solutions (Fig. 1, Table I) is similar to that found for Cu(II) tetrapeptide systems in which four nitrogens are able to coordinate to the metal ion [2, 8, 9]. The stepwise change of the d–d band energy towards higher values (*i.e.* lower wave length) results from the stepwise coordination of peptide nitrogens to Cu(II) ion. At low pH the Cu^{2+} ion coordinates to the MIF molecule through the deprotonated nitrogen atom of the N-terminal proline residue (1N complex where 1N labels the number of nitrogen donors bound equatorially to the metal ion). It subsequently binds the amide nitrogen atoms of the two peptide linkages (2N and 3N complexes). Above pH 8 the copper also coordinates to the amide nitrogen of the C-terminal amide group (4N complex) as shown below:

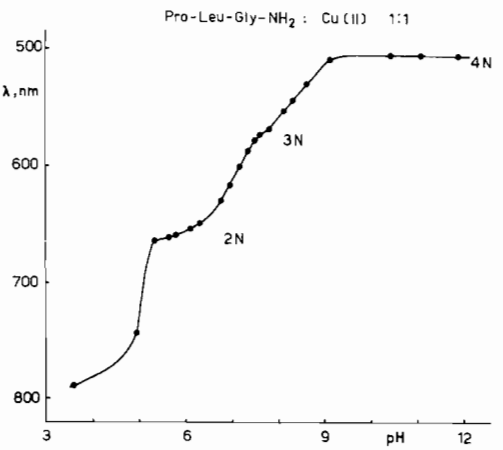


Fig. 1. The pH dependence of d–d band position for Cu(II): MIF 1:1 solutions.

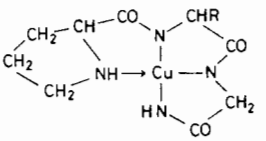


TABLE I. The Spectroscopic Characterization of Copper(II) Complexes with MIF Peptide.

Species	Vis.		CD		EPR	
	λ, nm	ε	λ, nm	Δε	$A_{\|\|}$, G	$g_{\|\|}$
40	800	10			131	2.367
					144	2.326
2N	660	40	695	−0.12	170	2.243
			565	−0.10		
			310	+0.44		
			260sh	−1.20		
			240	−1.50		
			207	+1.80		
3N	~570	80	580	−0.23	190	2.190*
			310	+0.47		
			273	−0.93		
			231	−0.71		
			207	+2.85		
4N	503	130	503	−0.36	212	2.163
			293	+0.58		
			260	−1.28		
			233sh	+0.50		
			207	+7.03		
MIF, pH 6–10			230	−0.08		
			206	+1.59		

*Very broad spectrum.

The binding of amide nitrogen atoms causes a characteristic increase in the molar absorbance (Table I, [9]). Both Fig. 1 and Table I show that absorption spectra are not able to characterise the 1N complex (*i.e.* the complex with only the proline nitrogen bound to the Cu(II) ion). Even EPR spectra cannot characterize the 1N species in this system, although this method is usually very sensitive in that respect [2, 4, 9]. This suggests a rather low concentration of the 1N species in the Cu(II)–MIF solutions. In the CD spectra only 2N, 3N and 4N species can be distinguished supporting this interpretation. Potentiometric studies (see below) clearly show that the 1N (or 1IO) complex is only a minor species in the Cu(II)–MIF equilibrium.

In the region of a d–d transition the CD spectrum of the 2N complex consists of two negative bands at 695 and 565 nm resulting from $B_{1g} \rightarrow B_{2g}$ (B) and $B_{1g} \rightarrow E_g$ (E) transitions respectively [2, 4, 10]. For 3N species one asymmetric negative band is observed at 580 nm (B + E) and for the 4N complex this broad negative signal shifts to 503 nm. The CD spectra of the 2N and 3N Cu(II)–MIF complexes resemble the spectra for the corresponding complexes of Cu(II) with Pro-Ala-Ala-Ala [2]. The 4N complex in the latter system is considerably different in its circular dichroism (four bands are observed in d–d region) compare with that of Cu(II)–MIF, most probably due to the fact that a different number of amino acid residues is bound to the cupric ion in the 4N complex.

The 2N, 3N and 4N complexes formed in Cu(II)–MIF solutions also exhibit two charge transfer transitions in the CD spectra at 290–310 nm (positive signal) and 260–270 nm (negative signal) (Table I). Similar charge transfer bands were observed for several Cu(II) di- and tripeptide systems [12], though their Cotton effects could have a reversed signs due to bulky amino acid side-chains [4]. The interpretation of the lower energy band at 310–340 nm region is generally accepted to be a N^- (peptide linkage) $\rightarrow$ Cu(II) charge transfer transition [4, 11, 13–15]. The assignment of the other band in the 260–280 nm region is more controversial. Garnier *et al.* [14, 15] have proposed that a negative signal in this region could be assigned to a N-terminal amine $\rightarrow$ Cu(II) charge transfer transition. Such an assignment seems to agree with our earlier study of the Cu(II)Ala-Ala-Ala-Ala and other related systems [2]. A comparison of the results obtained for the Cu(II)TRF system (TRF = L-pyroglutamyl-L-histidyl-L-prolinamide) [4] with those for copper complexes with TRF analogues [16], however, suggests that the CD extremum at 260–280 nm is compatible with coordination of the imidazole nitrogen to the Copper ion. Hence the appearance of this signal in systems containing proline on the peptide N-terminal (*i.e.* Cu(II)-MIF and Cu(II)Pro-Ala-Ala-Ala [2]) makes the above interpretations less convincing.

The MIF molecule exhibits two intraligand transitions in its CD spectrum, a positive band at 205 nm and a negative band at 230 nm. They result from $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions in the carbonyl chromophores.

Metal ion coordination to the MIF ligand causes a substantial increase in both of these Cotton effects. An interesting variation is observed when the 4N complex is formed from the 3N species (Table I). The band at 230 nm changes its sign from negative to positive. This suggests strongly that a considerable change in the conformation of the ligand molecule takes place when the C-terminal amide nitrogen binds to the metal ion, closing the fourth chelate ring around the metal. It may also suggest that the formation of a 4N complex in the Cu(II)Pro-Leu-Gly-NH_2 system leads to similar conformational changes to those found in the Cu(II)Pro-Ala-Ala-Ala system, as demonstrated by the CD spectra in the d–d region (see above and ref. [2]).

Ni(II)MIF solutions. In the solutions containing Ni(II) and MIF two symmetries of metal complexes are observed. Octahedral complexes are formed below pH 8 while square planar complexes are found above pH 8. The planar complex exhibits a d–d absorption band at 400 nm with a shoulder at 470 nm, which is characteristic of Ni(II) complexes with four nitrogens bound equatorially to the metal ion [9]. The CD spectra of this complex consist of two signals in the d–d region at 455 (–1.50) and 379 nm (+0.15). These two bands result from $A_{1g} \rightarrow A_{2g}$ and $A_{1g} \rightarrow E_g$ transitions respectively [3]. The intense positive Cotton effect at 257 nm (+5.08) originates from the amide nitrogen $\rightarrow$ Ni(II) charge transfer transition.

It was necessary to use a large ratio of ligand to Ni(II) (5:1) in order to prevent precipitation. As a result the intraligand transition range was entirely uninformative. It is likely, however, that the peptide conformation in the planar Ni(II)–MIF complex is close to that of 4N Cu(II)–MIF species.

Thermodynamic Studies

Cu(II)–MIF solutions. Calculated formation constants for the complexes formed with H^+, Cu^{2+} and Ni^{2+} are shown in Table II.

The hydrogen ion complex formation constant, log K_H (or pK_1) = 8.80 ± 0.03, is the mean value of five titrations using two independent samples of ligand and individually weighed quantities in each titration. Within a particular titration the standard deviation was $< \pm 0.02$. The only value reported in the literature is log K_H = 8.40 [17] but no experimental details are given. The formation constant must refer to protonation of the proline nitrogen atom. This will be considerably more acidic than in proline itself (log K = 10.5) since it has no neighbouring CO_2^- group. Prolylglycine has a value of 8.98 [18], closely comparable to that of MIF.

TABLE II. The Formation Constants of Complexes of MIF with H^+, Cu(II) and Ni(II) at 25 °C and $I = 0.10\ M$ ($K[NO_3]$). Log β_{xyz} Values for the Complexes $M_xL_yH_z$. (Standard deviations are given in parentheses).

H^+	$\log \beta_{011}$ =	8.80(3)	5 titrations
Cu(II)	1N: $\log \beta_{110}$ =	5.69(2)	2 titrations
	2N: $\log \beta_{11-1}$ =	0.02(1)	
	3N: $\log \beta_{11-2}$ =	−7.23(2)	
	4N: $\log \beta_{11-3}$ =	−16.30(2)	
Ni(II)	4N: $\log \beta_{11-3}$ =	−18.1(1)	2 titrations

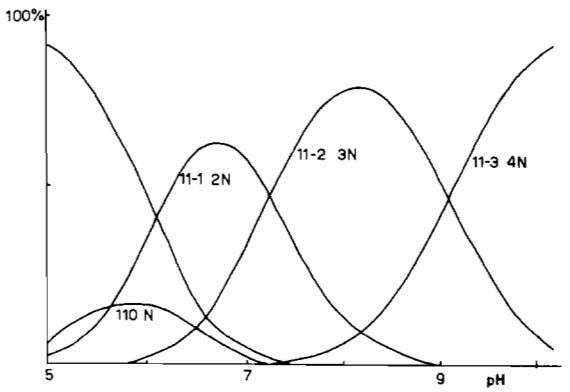


Fig. 2. The species distribution curve for Cu(II):MIF 1:1 solutions.

With Cu(II) four complex species are formed between pH 4 and 10. These are shown on the species distribution curve for a 1:1 mixture given in Fig. 2. The first complex to form, $[CuL]^{2+}$, is only a minor species existing between pH 4.5 and 7. It never coordinates more than about 15% of the Cu(II), so supporting the spectral evidence given earlier. This species is replaced successively as the pH is raised by the deprotonated complexes $[CuLH_{-1}]^+$ (11 – 1 species, pH 5.5 to 8), $[CuLH_{-2}]$ (11 – 2 species, pH 6–10) and $[CuLH_{-3}]^-$ (11 – 3 species, pH 8 upwards). The number of protons displaced corresponds to the deprotonated nitrogen atoms able to coordinate to the Cu(II) in addition to the proline nitrogen. Hence the 11 – 3 species will be the 4N complex of Fig. 1. This was confirmed by the bright pink colour which developed above pH 9, when the 11 – 3 species becomes the predominant complex [8]. A comparison of Figs. 1 and 2 demonstrates the agreement between the spectroscopic and potentiometric evidence.

Values for the stepwise protonation constants for the 4N complex ($[CuLH_{-3}]^-$) may be derived from the results in Table II. Values obtained are $\log K^{11-2}_{11-3} = 8.07$, $\log K^{11-1}_{11-2} = 7.27$ and $\log K^{110}_{11-1} = 5.67$. The corresponding values for prolylglycine are $\log K^{11-1}_{11-2} = 9.57$ and $\log K^{110}_{11-1} = 4.01$.

Ni(II)–MIF solutions. Complex formation between Ni(II) and MIF was found to be exceedingly slow at 25 °C, and 1:1 metal:ligand solutions tended to precipitate (*cf* spectroscopic studies). Even with a 1:5 ratio equilibrium required up to 30 minutes per titration point, introducing many experimental problems. As a result the experimental data below pH 8 could not be used satisfactorily. However, above pH 8 a $[NiLH_{-3}]^-$ complex could be identified clearly (the planar complex identified spectroscopically). It is therefore reasonable to assume that this is a 4N complex comparable to the Cu(II) analogue.

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