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Copper(II) Complexes of Oligopeptides. An Equilibrium and Spectroscopic Study on the Copper(II) Lys-Leu-Ala-His-Phe-Gly System

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The hexapeptide L-Lys-L-Leu-L-Ala-L-His-L-Phe-Gly was prepared by solid-phase peptide synthesis. The proton and copper(II) equilibrium system and thereby the composition and solution structure of the complexes formed were investigated by potentiometric and spectrophotometric titrations, as well as by CD and EPR spectroscopy. The ligand was able to keep two equivalents of metal ion in solution in the investigated pH interval 2–10. While the protonation isomers form separately, the formation of the metal complexes of different protonation states can be characterized by overlapping processes. The detectable coordination starts at ca. pH 3–4, where, beside the possible weak carboxylate coordination, the parallel deprotonation of the imidazole nitrogen atom and the *N*-terminal L-lysine α-ammonium group takes place in the presence of metal ions. In this way a large chelate ring is formed. The first significant signals in the CD and EPR spectra can be attributed to the species $[ML]^+$, with one deprotonated amide nitrogen in addition to the abovementioned donor groups. A species with four coordinating nitrogen atoms containing electrically neutral the MLH_{-1} complex is predominant in the physiological pH region. The spectroscopic results strongly suggest that there are at least two deprotonated amide nitrogens in the copper(II) coordination sphere. The formation of the next species requires the replacement of one of the already coordinated donor groups (probably the imidazole nitrogen). The drastic changes in the absorption and CD spectra and the increase of the A_0(Cu) to 92 G support the assumed processes. The deprotonation above pH 9.5 proved to be due to the *N*-terminal L-lysine ε-ammonium group, which remains uncoordinated even under favorable conditions. The formation of binuclear species with well separated copper(II) centers was detected by EPR spectroscopy when an excess of metal ion was applied.

It has been recognized for a long time that copper is an element of basic importance for proteins involved in living processes. It is known that some copper(II) ions (e.g. in blood) are exchangeable *in vivo* and are associated with albumin and low-molecular-weight peptides containing L-histidine. The function of these coordination compounds depends on the ligand donor group set and the geometry of the coordination sphere, i.e. on the coordination structure of the copper complex formed in the protein matrices, also determining the structure of the peptide. A number of crystal structure determinations and attempts to investigate solutions of copper(II)-containing proteins can be found in the literature.[1–4] However, the exact equilibrium study of natural proteins in an aqueous medium, which could clarify the thermodynamic stability and composition of the species in the physiological pH region, is difficult (problems of synthesis or isolation, molar mass, solubility, etc.). Therefore, small peptides are used as models for the active site of the macromolecules.

Recently evidence has begun to accumulate supporting the idea that peptides may be among the important mediators of human allergic disease. Structure–activity studies have been performed on a series of naturally

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occurring and synthetic polypeptides, by measurement of the polypeptides' ability to induce selective histamine release from normal rat peritoneal mast cells *in vitro*. Compounds investigated include corticotrophin and melittin derivatives, mast-cell-degranulating peptide from bee venom, polymyxin B, bradykinin and various synthetic polyamino acids and short-chain peptides.[5,6] It was found that histamine-releasing activity is increased by cyclization of the linear peptide hormones angiotensin, bradykinin, kallidin and neurotensin. In order to find peptides possessing histamine-releasing activity several linear and cyclic analogues of the ACTH active center were investigated, and induction of histamine release from mast cells by cyclic analogues was observed.[7]

Metal ions may act synergistically with peptides promoting their biological activity, as was shown, e.g., for Gly-L-His-L-Lys, a growth-modulating tripeptide from human plasma.[8] Very recent results show that the coordination chemistry of such compounds has not yet been fully clarified, and that special attention is directed towards transition metal complexes of the histidine-containing peptides.[9]

For the reasons described above, we prepared a number of hexapeptides with different amino acids and different amino-acid sequences in open-chain and cyclic form, which can also be considered as modified analogues of the ACTH active center. The hexapeptide L-Lys-L-Leu-L-Ala-L-His-L-Phe-Gly was chosen first to investigate the equilibrium processes and solution structure of its copper(II) complexes to determine the stability and the composition of the complexes formed under various conditions.

Experimental

Materials and methods. All solvents were distilled at the ambient pressure. DMF was analyzed for free amines by addition of Dhbt-OH prior to use. Reagents for peptide synthesis were purchased as follows: Dhbt-OH, HOBT and TBTU from Fluka, Fmoc-amino acids, Fmoc-Gly-NovaSyn KA resin from Novabiochem.

HPLC was performed on a Waters HPLC system with a C_{18} reversed-phase column (5 μm, flow rate 1.5 ml min^{-1} for analytical separations) with a buffer A (0.1% TFA in water) and a buffer B (0.1% TFA and 10% water in acetonitrile). Amino acid analysis was performed on a Pharmacia LKB Alpha Plus amino acid analyzer after hydrolysis of the peptide with 6 M HCl at 383 K for 24 h.

The copper(II) perchlorate, used for the pH metric measurements was Fluka product. The concentration of the standard metal-ion solution was determined complexometrically.

Abbreviations: ACTH, adrenocorticotropic hormone; Boc-, *tert*-butyloxycarbonyl; Dhbt-, 3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl; DIEA, diisopropylethylamine; DMF, dimethylformamide; Fmoc-, 9-fluorenylmethoxycarbonyl; HOBT, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TMPA, tris[(2-pyridyl)methyl]amine.

Synthesis of hexapeptide L-Lys-L-Leu-L-Ala-L-His-L-Phe-Gly. The hexapeptide was synthesized by a solid-phase method in a syringe supplied with an Omnifit Teflon filter (100 μm, 2 mm thick). To the syringe was added 2.1 g NovaSyn KA (substitution 0.08 mmol/g) with the appropriate first Fmoc-protected amino acid (Gly) coupled through the acid labile linker 4-hydroxymethylphenoxyacetic acid. The resin was swollen in DMF for 30 min and then deprotected with 20% piperidine containing 0.01% azoruby in DMF (2 ml) for 10 min. The last step was repeated. The resin was inspected visually during the deprotection to ensure even distribution of azoruby, indicating distribution of piperidine. The resin was washed with DMF (10 × 40 ml). At this stage all traces of azoruby had disappeared, indicating complete removal of piperidine.[10]

The second amino acid was coupled as Fmoc-protected L-Phe (4 equiv.), activated by means of TBTU (4 equiv.) and promoted by HOBT (4 equiv.) (0.2 M DMF solution) 6 equiv. of DIEA was added.[11] The coupling was left overnight. Afterwards the resin was washed with DMF (10 × 40 ml) followed by deprotection with piperidine as described above.

The next amino acids were coupled by the same protocol. L-Lys and L-His were used as Fmoc-L-Lys(Boc)-OH and Fmoc-L-His(Boc)-OH derivatives. After the last Fmoc deprotection and washing with DMF, resin was carefully washed with diethyl ether (3 × 40 ml) and left to dry. The dry resin was transferred to glass tube for TFA cleavage. 95% TFA in water was added to the tube, and the mixture was left for 2 h. The TFA solution was filtered, the resin was washed with 95% TFA (3 × 20 ml), and all four extracts were mixed and evaporated to dryness. The resulting peptide was extracted three times for 10 min with diethyl ether and finally dried cautiously. The dry peptide was dissolved in 30 ml water and lyophilized with a yield of 151 mg (96.5%). HPLC analysis indicated a presence of a single peak. The results of amino acid analysis yielded: Gly 1.01; His 1.05; Ala 0.95; Leu 1.01; Phe 1.01; Lys 0.98.

pH-Metric measurements. Both the protonation and metal ion coordination equilibria were investigated by potentiometric titrations in aqueous solutions. The ionic strength was adjusted to 0.1 M with $NaClO_4$ and the cell was thermostatted to 298.0 ± 0.1 K. In order to remove carbon dioxide and molecular oxygen, high-purity nitrogen gas was bubbled through the solution during the titration. The combined glass electrode (Metrohm 6.0202.110) was calibrated by means of standard buffer solutions. The pH values were recorded by a digital Metrohm 691 pH meter in a full automatic titration set, where Metrohm 665 dosimat was used for addition of standardized NaOH solution. The species formed in the systems studied can be characterized by the general equilibrium process (1), while the formation constants

for these generalized species are given by eqn. (2). (Charges are omitted for simplicity.)

$$pM + qL \xleftrightarrow{\beta_{M_pL_qH_{-r}}} M_pL_qH_{-r} + rH \quad (1)$$

or

$$pM + qL + rH_2O \xleftrightarrow{\beta_{M_pL_q(OH)_r}} M_pL_q(OH)_r + rH$$

$$\beta_{M_pL_qH_{-r}} = \beta_{M_pL_q(OH)_r} = \frac{[M_pL_qH_{-r}][H]^r}{[M]^p[L]^q}$$

$$= \frac{[M_pL_q(OH)_r]K_W^r}{[M]^p[L]^q[OH]^r} \quad (2)$$

The equilibrium constants were determined from five independent titrations in each system with the computer program PSEQUAD.[12] The metal-to-ligand ratios were varied from 2:1 to 1:2 and the metal ion concentrations ranged from 5×10^{-4} to 1×10^{-2} M.

EPR measurements. The EPR spectra were recorded on a JEOL JES-FE 3X spectrometer in the X-band at 298 K with 100 kHz field modulation at room temperature. Manganese(II)-doped MgO powder served as field standard. The copper(II) concentration was 5×10^{-3} M.

Electronic absorption and CD measurements. UV/VIS spectra were recorded on a Hewlett Packard 8452A diode array spectrophotometer, while CD spectra were recorded on a Jasco J-710 spectropolarimeter in the wavelength interval from 180 to 800 nm. The metal ion concentration was 5×10^{-4} M in a cell with 0.1 or 1 cm optical pathlengths. CD data are given as the differences in molar absorptivities between left and right circularly polarized light based on the metal ion concentration ($M^{-1}\,cm^{-1}$).

Results and discussion

Potentiometric titrations. Protonation constants were determined from five independent pH-metric titrations. The hexapeptide investigated contains four donor groups, which deprotonate with increasing pH in the pH 2–10 interval. These are the *C*-terminal glycine carboxyl, the L-histidine imidazole nitrogen, the *N*-terminal L-lysine α-ammonium and the *N*-terminal L-lysine ε-ammonium groups in increasing order of the p*K* values (Table 1). Since the species of well determined protonation states form separately during the titration, the assignment of the p*K* values to individual groups is fairly straightforward on the basis of the literature of amino acids and peptides.[1] According to the species distribution diagram (Fig. 1) the fully protonated $[LH_4]^{3+}$ species dominates at low pH and it completely disappears after pH 5. In the physiological pH region the species $[LH_2]^+$ dominates while the species L^- becomes the major species only above pH 10.

Investigations with copper(II) ion revealed that the ligand is able to keep 2 equiv. of metal ion in solution in the whole pH interval studied. Thus the ligand to metal ratio was varied from 2:1 to 1:2 during the potentiometric titrations. The curve-fitting process strongly suggested that in the systems with L:M = 2:1 and 1:1 the same species are formed, i.e. complexes containing the metal ion and the ligand in equivalent amounts and differing from each other only in their protonation states. It means that there is no space for two bulky ligands around the copper(II) ion. However, in the systems with L:M = 1:2, completely different binuclear species are formed. The computed overall stability constants are shown in Table 1.

From the species distribution diagram of the L:M = 2:1 system (Fig. 2a) one can see that the first complex appears in significant amounts only above pH 3–4. As usual for peptides, the stability of the species $[MLH_3]^{4+}$ coordinated by the carboxylate group only is too low, thus the first detectable coordination species is $[MLH_2]^{3+}$. On the basis of the stability constant of this species derived from the equilibrium process

$$M^{2+} + LH_2^+ \xleftrightarrow{K_{MLH_2^{3+}}} MLH_2^{3+}$$

as $\log K_{MLH_2^{3+}} = \log \beta_{MLH_2^{3+}} - \log \beta_{LH_2^+} = 4.52$ it is difficult to distinguish between the terminal amino and the imidazole nitrogen coordination. This constant is very close to the copper(II) complexes of tetrapeptides with *C*-terminal L-His residue (ca. 4.5),[13] where besides the imidazole coordination chelation through the carboxylate group is possible. In our case the peptide carbonyl oxygen is in the same chelating position. However, the stability constants of copper(II) peptide complexes (containing amino acids with non- or weakly coordinating side chains only) at low pH, i.e. when they are coordinated by the terminal amino group with possible chelation through the carbonyl oxygen, vary from 4.6 to 5.9.[2] On the basis of the above considerations the most obvious is to suggest the parallel formation of either amine and imidazole coordinated 'microspecies' as was also done for L-glycylhistidine.[14]

The $pK_{aMLH_2^{3+}}$ of 5.21 (Table 1) corresponds to the acid/base equilibrium between the complexes $[MLH_2]^{3+}$ and $[MLH]^{2+}$. This value could be assigned either to the deprotonation of the so far non coordinated L-lysine α-ammonium or of the protonated imidazole nitrogen, in this way increasing the stability of the complex up to $\log K_{MLH_2^+} = 6.86$. The deprotonation of the amide nitrogen neighboring the primarily coordinated donor group also can not be excluded.

The next deprotonation process with $pK_a = 5.92$ yields the species $[ML]^+$. This value is low in comparison with the pK_a values of the copper(II) tri- or tetraglycine complexes (6.60 and 6.89, respectively) corresponding to the second peptide nitrogen deprotonation. This strongly suggests that our species does not contain two deprotonated peptide groups. Thus we conclude that the three deprotonated donor groups in this complex are the terminal amine, the imidazole nitrogen atom and one of

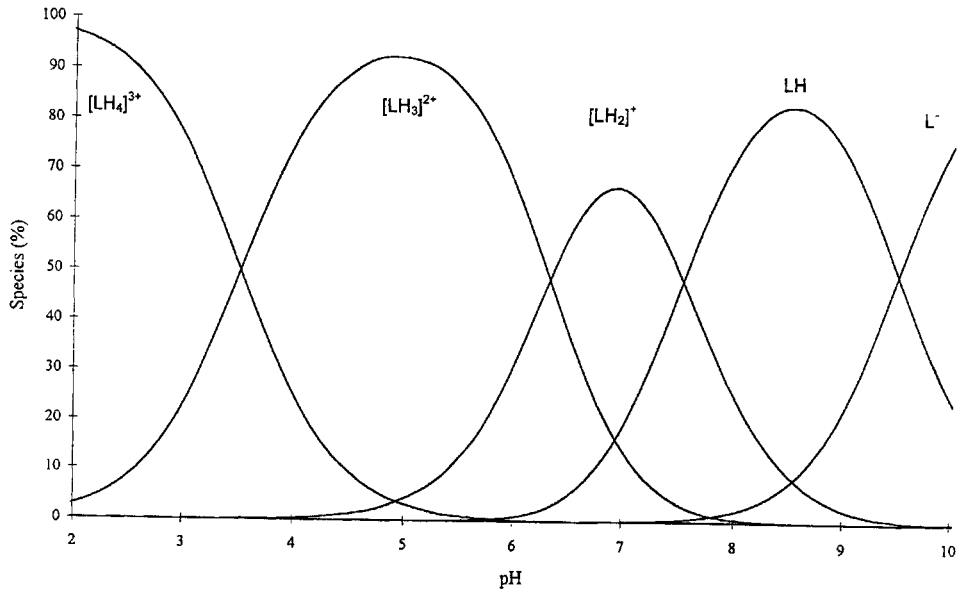

Fig. 1. The distribution of the species of different protonation states as a function of pH in 0.005M solution of L-Lys-L-Leu-L-Ala-L-His-L-Phe-Gly hexapeptide (I=0.1 M $NaClO_4$, T=298 K).

Table 1. Overall stability constants for the proton and copper(II) complexes of the linear hexapeptide L-Lys-L-Leu-L-Ala-L-His-L-Phe-Gly in aqueous solutions (I=0.1 M, $NaClO_4$; T=298 K).

p	q	r	log $\beta_{M_pL_qH_r}$	log $K^{M_pL_qH_r}_{M_pL_qH_{r-1}}$ [a]
0	1	4	26.95 (3)	3.53
0	1	3	23.42 (3)	6.34
0	1	2	17.08 (2)	7.55
0	1	1	9.53 (2)	9.53
1	1	2		5.21
1	1	1	16.39 (7)	5.92
1	1	0	10.47 (8)	6.95
1	1	−1	3.52 (8)	8.94
1	1	−2	−5.42 (10)	10.06
1	1	−3	−15.48 (13)	
2	1	1	19.41 (5)	5.68
2	1	0	13.73 (5)	6.11
2	1	−1	7.62 (7)	6.77
2	1	−2	0.85 (7)	7.50
2	1	−3	−6.65 (8)	8.78
2	1	−4	−15.43 (9)	9.69
2	1	−5	−25.12 (11)	

[a] $K^{M_pL_qH_r}_{M_pL_qH_{r-1}}$ refers to the equilibrium $M_pL_qH_{r-1}+H \leftrightarrow M_pL_qH_r$; thus, log $K^{M_pL_qH_r}_{M_pL_qH_{r-1}}$ = log $\beta_{M_pL_qH_r}$ − log $\beta_{M_pL_qH_{r-1}}$.

the peptide nitrogen atoms. The value for the absorption max, λ_{max}=618 nm, for this species* also supports the latter assumption, taking into account the very similar value (610 nm) measured in the copper(II) Gly-L-His-L-Lys system,[15,16] where the same donor groups are coordinated to the metal ion as determined by X-ray crystallography.[17] The empirically calculated[18] value is 599 nm, which would be even 20 nm less assuming two deprotonated amide nitrogens.

* The wavelengths of the absorption maxima were calculated by the computer program PSEQUAD.

Only the electrically neutral complex MLH_{-1} formed with increasing pH is stable in a relatively wide pH range. The copper(II) coordination sphere is saturated in this species with four donor groups in equatorial positions, since one more peptide nitrogen became deprotonated. This assumption corresponds to the pK_a=6.95, which is very close to the value due to the deprotonation of the second peptide nitrogen in simple tri- or tetrapeptides. The shift of the absorption maximum to 544 nm also supports the above consideration (the calculated value on the basis of Billo's results[18] is 540 nm). The increased stability can be attributed to the fact that the next coordinating donor group has to replace one of the already coordinated groups.

At higher pH the situation may be similar to that described in the literature for the peptide angiotensin II,[19] where copper(II) is proposed to move from the imidazole nitrogen to the terminal amine end of the peptide with increasing pH. Presumably an another deprotonated amide nitrogen (pK_a=8.94) will replace the imidazole nitrogen, yielding a very stable complex $[MLH_{-2}]^-$ in alkaline medium with λ_{max}=504 nm (the calculated value with three amide nitrogens and the terminal amine groups coordinated is 519 nm[18]).

The next deprotonation (resulting in the species $[MLH_{-3}]^{2-}$) has no effect on the structure of the complex formed, as seen from the CD and EPR measurements (see later). It must in fact be due to the deprotonation of the non-coordinated ε-ammonium group of L-lysine. The pK_a value for this process is 10.06, a little higher than the corresponding value of 9.53 in the free ligand. This may be attributed to the increased negative charge, i.e. negative inductive effects in the complex molecule. As it is proposed in the literature of

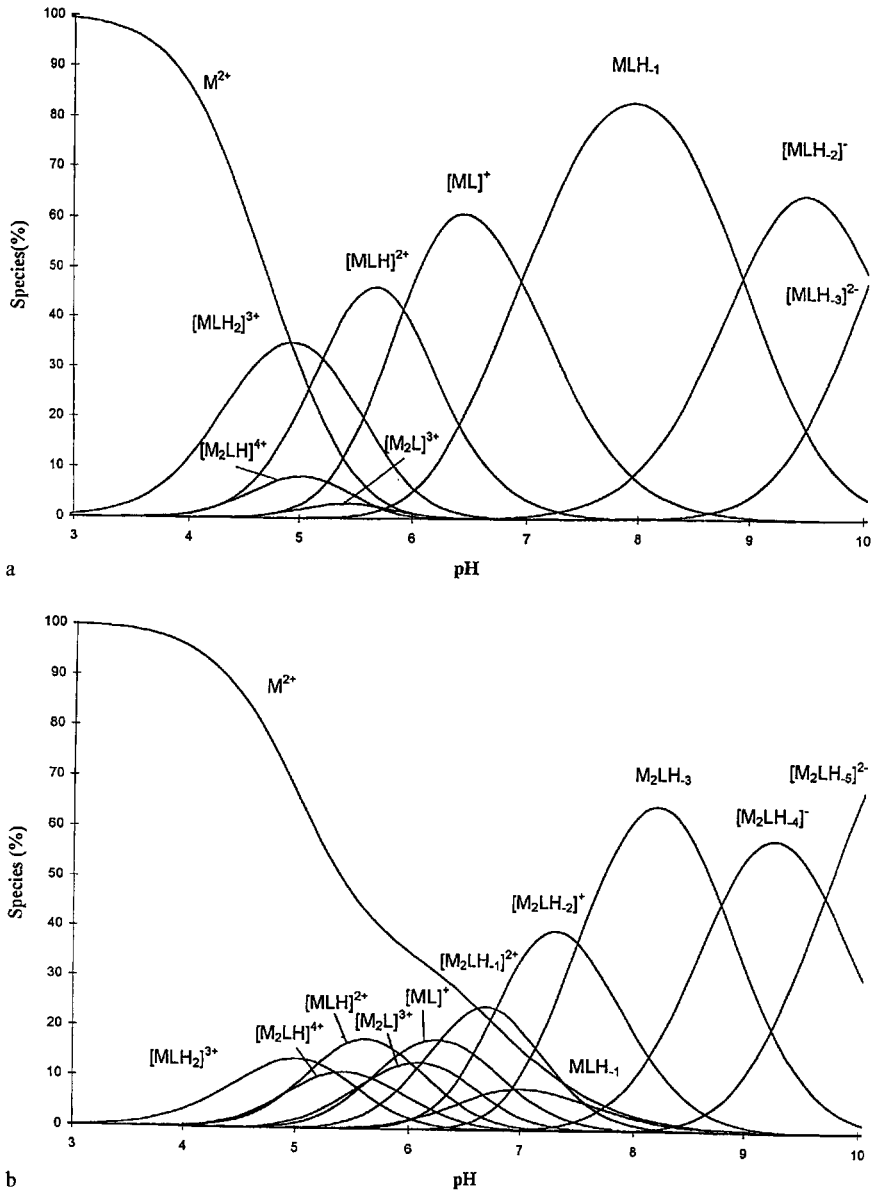

Fig. 2. Species distribution diagram for the copper(II): L-Lys-L-Leu-L-Ala-L-His-L-Phe-Gly hexapeptide systems (I=0.1 M, $NaClO_4$, T=298 K). (a) L:M=2:1, $C_{Cu^{2+}}=8\times10^{-4}$ M. (b) L:M=1:2, $C_{Cu^{2+}}=8\times10^{-4}$ M.

L-lysine-containing peptides, this group is in the vast majority not complexed.[15,17] The wavelength of the absorption maximum remained also the same as for the species $[MLH_{-2}]^-$.

The investigation of the systems with an L:M ratio 1:2 seems to be much more complicated. As the species distribution diagram shows, binuclear species were dominating in the whole pH interval. The first detectable binuclear species above pH 4 ($[M_2LH]^{4+}$) has been proposed to be coordinated by the *N*-terminal L-lysine α-amine group to one and by the imidazole nitrogen to another copper(II) ion. Several deprotonation processes follow with the increase in pH. However, the pK_a values are significantly lower than those for the mononuclear complexes, indicating the smaller number of the deprotonated groups around an individual copper(II) ion, i.e. the higher positive charge of the species. The pK_a values suggest that the deprotonations take place parallel in the surroundings of both metal ions coordinated to the peptide. This is also supported by the species distribution, since there are strongly overlapping equilibria in the whole pH region. This means that we always have two slightly different copper(II) centers, and it makes the evaluation of the spectrophotometric curves much more difficult. However, it is worth mentioning that above pH 8.5 the wavelength corresponding to the absorption maximum reaches the same value of 504 nm as for the species $[MLH_{-2}]^-$; the only difference is the significant asymmetry (presumably a shoulder at higher wavelengths) of the absorption band. Therefore we suggest that in the species $[M_2LH_{-4}]^-$ one

of the two copper(II) ions is coordinated by the *N*-terminal amino group and three deprotonated amide nitrogen groups ($\lambda_{max,calc} = 519$ nm), while the second by imidazole nitrogen and two more deprotonated amide nitrogens ($\lambda_{max,calc} = 574$ nm). If the coordination in the latter species has started toward the *C*-terminal end of the amino acid, then we may assume that the fourth equatorial position around the second copper(II) ion is occupied by the carboxylate oxygen.

Theoretically there is a possibility for the displacement of the glycine carboxylate group by the hydroxide ion above pH 10, but the measured pK_a value of 9.69 is lower than one would expect on the basis of the literature. The pK_a for the deprotonation of a coordinated water molecule, when only one negatively charged amide nitrogen is coordinated besides two neutral nitrogen donor atoms, is around 9.3–10.2.[14,20] However, this is not the case in our complex. If the number of the deprotonated amide nitrogens increases (ca. 10.4–10.9),[21] or if there are other negatively charged ligands around the copper(II) (ca. 10.5),[22] or the hydroxide ion has to replace a carboxylate group (ca. 11.9),[23] the pK_a values increase to more than 10. It is therefore reasonable to assume that the last deprotonation measured by us is due to the ε-ammonium group of the *N*-terminal L-lysine.

Circular dichroism measurements. A very important observation was that both the electronic absorption and the CD spectra of the copper(II)–hexapeptide systems in L:M = 2:1 and 1:1 composition showed almost the same pattern over the whole pH region studied, irrespective of the ligand to metal ratio. As in the case of potentiometric titrations, however, a difference was detected between the latter and the L:M = 1:2 systems. Figures 3a and 3b show the CD curves measured in the two different systems (L:M = 2:1 and 1:2, respectively) as a function of pH.

From Fig. 3a it can be seen that the circular dichroism intensity of the d–d bands for the copper(II) entities changes dramatically with pH. Making use of the species distribution (Fig. 2a) we have estimated the molar CD for each species. The complex formed at pH 3.6 gives a CD which is hardly noticeable. This is in agreement with the fact that the coordination takes place through the unidentate carboxylate group, which is achiral to the first approximation.

The first, potentiometrically detectable coordination around pH 5.0, where according to the species distribution diagram (Fig. 2a) complex $[MLH_2]^{3+}$ predominates, the Cotton effect is still practically absent. This is to be expected if the ligand is coordinated through its imidazole nitrogen only, far from the next chiral carbon atom. At ca. pH 5.7 the first chelate coordination produces a CD of quite low intensity both in the d–d and in the charge-transfer region, and it indicates to us that a large flexible chelate ring exists. The CD of the peptide region (not shown in Fig. 3) is also of remarkably low intensity, indicating a very low degree of ordering in the peptide backbone. Each conformer will produce a Cotton effect, and these contributions partly cancel on summation. However, at this pH about 20% of complex $[ML]^+$ is already formed, probably causing the transitions to have a more intense CD. The minimum at $\lambda \approx 660$ nm may be characteristic for the complex $[ML]^+$ (taking into account the absorption maximum). The pK value from the CD intensities graphically determined at several wavelengths near the extremum is 5.9 ± 0.1, in very good agreement with the pH metric results for the $ML^+ + H^+ \rightleftharpoons MLH^{2+}$ equilibrium.

The next CD curve at pH 6.6 shows that, in addition to the now dominating $[ML]^+$ species, a smaller amount of a new one is present in solution. This is presumably the 4 N species MLH_{-1} with two deprotonated amide nitrogens, which becomes predominant at pH 7.4. A complex with five-membered chelate rings emerges showing a negative CD couplet with the inflection point at about 530 nm. Since the species exhibit exciton-coupled transitions, we suggest that the structure of the π-systems of the chelate rings is non-coplanar, like the Schiff base complexes of copper(II).[24] The assignment of very intense charge-transfer transitions to the imidazole nitrogen, amino nitrogen or peptide nitrogen to copper(II) is ambiguous because of their overlapping[25,26] and partial cancellation.

At higher pH (>9) we see the CD gaining intensity in the d–d region. The very large negative Cotton effect at $\lambda \approx 510$ nm ($\Delta\varepsilon \approx -1.6$), corresponding to the copper(II) complexes containing three deprotonated peptide nitrogens suggests a biuret type of chelation. The shift in the charge transfer bands is presumably due to the decomposition of the copper(II) imidazole–*N* bond, which is replaced by a deprotonated amide nitrogen. The increase of the pH to above pH 10 does not significantly affect the shape of the CD spectra. From the calculations based on the computed species distribution we find that the two complex ions $[MLH_{-2}]^-$ and $[MLH_{-3}]^{2-}$ have the same molar CD, and the obvious explanation for this is that they differ in a way that is insignificant for the chromophores. The L-lysine ε-ammonium-to-amine equilibrium seems to be such an optically insignificant proton equilibrium, supporting the idea that the species $[MLH_{-2}]^-$ is structurally the same as $[MLH_{-3}]^{2-}$; in the latter the non-coordinating ε-ammonium group is deprotonated.

Figure 3b shows the CD spectra of the system with L:M = 1:2 at different pH values. A very nice indication of the formation of similar coordination spheres in binuclear complexes as in the L:M = 2:1 system can be seen. The differences between the two systems are the formation of the binuclear species at lower pH (according to the lower pK values and higher positive charge of these species), the asymmetry of the CD bands, indicating that there are always two different copper(II) centers in the solution (i.e. binuclear complexes), and the significantly lower intensity of the charge-transfer bands in the former system, which comes from the larger cancellation effect of the increased number of the copper(II) centers.

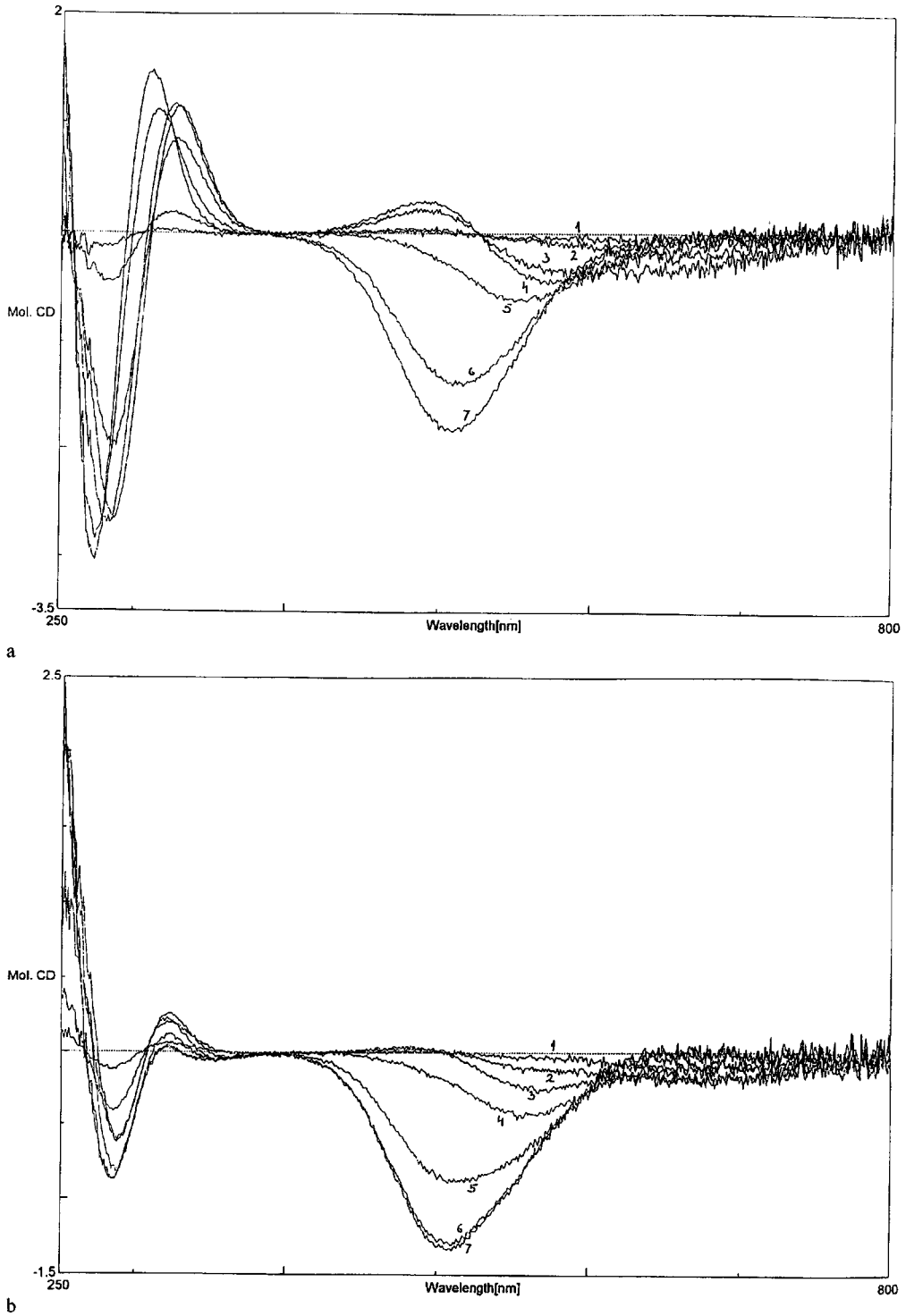

Fig. 3. CD curves of the copper(II) L-Lys-L-Leu-L-Ala-L-His-L-Phe-Gly systems with different L:M ratios as a function of pH. (a) L:M = 2:1, $C_{Cu^{2+}} = 5 \times 10^{-4}$ M, (1) pH 5.0, (2) pH 5.7, (3) pH 6.6, (4) pH 7.4, (5) pH 8.4, (6) pH 9.4, (7) pH 10.5; (b) L:M = 1:2, $C_{Cu^{2+}} = 5 \times 10^{-4}$ M, (1) pH 5.5, (2) pH 6.1, (3) pH 6.6, (4) pH 7.4, (5) pH 8.4, (6) pH 9.7, (7) pH 10.2.

Electron paramagnetic resonance spectroscopy. The EPR spectra for the systems with L/M = 1:2 as a function of pH were recorded at room temperature. Because of the overlapping formation of the different complexes, we chose the pH values for the EPR measurements from the species distribution diagram (Fig. 2a) in such a way that no more than two species may exist in significant amount in solutions. Then the spectra were evaluated by a computer program,[27] and the parameters obtained are listed in Table 2. In the pH region studied no visible

nitrogen superhyperfine splitting can be obtained, as a consequence of the very broad copper(II) hyperfine lines. Such a line-broadening effect is caused, according to the anisotropic magnetic relaxation theory in solution,[28] by the large rotation correlation time, which is proportional to the molecular radius of the equivalent rotating sphere. The widths of lines between states with smallest nuclear magnetic quantum numbers are the most informative, since they reflect that with increasing pH the ligand is coordinated with more donor groups; thus, it comes closer and closer to the metal ion, decreasing the radius of the molecule. Unfortunately our hexapeptide ligand is still too large to obtain well resolved EPR spectra.

Nevertheless, the spectra reflect the same tendency as the CD spectra. The isotropic hyperfine copper(II) coupling constants (Table 2) increase, while g_0 values decrease as the pH increases, indicating that there are more and more amide nitrogens in the equatorial plane of the copper(II) coordination sphere. The spectra between pH 6.5 and 7.7 can be fitted assuming the species $[ML]^+$ and MLH_{-1} with three and four nitrogen donor atoms, respectively. The value $A_0 = 55$ G for the former complex seems to be very small in comparison with the 70–75 G usually determined for the peptide complexes containing three nitrogens.[15,20,29,30] In these complexes, however, the nitrogen donor groups are the members of fused five membered chelate rings. The species $M(LH_{-1})L$ containing three nitrogens in copper(II) glycyl–glycine system also shows very similar parameters, $g_0 = 2.116$ and $A_0 = 55$ G. The authors proposed a structure for this species in which besides the terdentate coordination of the first ligand in the equatorial plane, the fourth equatorial site and one of the axial sites are occupied by the carbonyl oxygen of the amide group and the amino nitrogen, respectively. The wavelength of the absorption maximum was also determined by spectrophotometric measurements at 625 nm. If we, however, use the so-far satisfactory equation[18] to predict λ_{max} it will be around 680 nm for this structure. Better agreement can be achieved with the amino nitrogen in the equatorial position and the carbonyl oxygen in the axial position (622 nm), which could also be a reliable structure. In our case $\lambda_{max} = 618$ nm and the fit of the EPR spectra assuming three nitrogen donor atoms suggest some similarities to the latter complex of glycyl–glycine, i.e. in both species only two nitrogen donor atoms are members of a five membered chelate ring and the third one binds 'separately'. This is in agreement with our previous assumption concerning the coordination of the *N*-terminal L-lysine α-amine, one of the deprotonated amide nitrogens and the imidazole nitrogen in $[ML]^+$.

The parameters determined for the species MLH_{-1} are in very good agreement with the parameters determined for the CuN_4 species, with at least two deprotonated amide nitrogen atoms. The contribution of the species to the EPR spectra is reflected by the species distribution also obtained from the fitting procedure (Table 2), in good agreement with the potentiometric measurements. Figure 4 shows as an example the analysis of the spectrum measured at pH 6.9. At pH 6.5 the species $[MLH]^{2+}$ can not be detected owing to its small concentration and its uncharacteristic EPR spectrum.

A further increase in pH results in a new EPR spectrum at pH 9.6, having a larger A_0 and smaller g_0 than the former. This is a very nice proof that the two species containing four nitrogens differ in the quality of these donor atoms, i.e. the imidazole nitrogen in species MLH_{-1} is replaced by a deprotonated amide nitrogen in species $[MLH_{-2}]^-$. This, and the following spectrum at pH 10.3 can be fitted assuming only one complex species in solution, in contrast to the species distribution curve from potentiometric titrations (Fig. 2a). As Table 2 shows, the parameters determined for the two spectra are the same within experimental error, like the CD spectra detected in the same pH interval. This again supports the result that during the formation of the complex $[MLH_{-3}]^{2-}$ the deprotonation of the *N*-terminal L-lysine ε-ammonium group occurs, having no effect on the EPR parameters.

EPR spectra were also detectable in the L:M = 1:2

Table 2. The isotropic *g*-values, copper(II) hyperfine coupling constants, nitrogen superhyperfine coupling constants, number of the coordinated nitrogen donor atoms and the linewidths determined from the EPR spectra of the copper(II)–L-Lys-L-Leu-L-Ala-L-His-L-Phe-Gly 1:2 system at different pH values.[a]

pH	Species	(%)[c]	g_0	A_0/G	A_0(N)/G	n_N	σ_M/G			
							−3/2	−1/2	1/2	3/2
6.5	$[ML]^+$	(78)	2.111	55.0	9	3	180	93	45	35
	MLH_{-1}	(22)	2.090	86.3	10	4	148	71	45	33
6.9	$[ML]^+$	(49)	2.111	55.2	10	3	181	87	48	36
	MLH_{-1}	(51)	2.090	86.3	10	4	141	77	42	32
7.7	$[ML]^+$	(16)	2.111	54.9	10	3	185	94	50	36
	MLH_{-1}	(84)	2.090	86.1	10	4	144	72	45	33
9.6	$[MLH_{-2}]^-$	(100)	2.085	91.8	11	4	121	59	36	28
10.3	$[MLH_{-3}]^{2-}$	(100)	2.084	92.0	11	4	115	58	36	28

[a]The metal ion concentration was 5×10^{-3} M, $T = 298$ K. g_0-values are considered to be accurate to ±0.002, A_0-values to ±0.2 G and the linewidth to ±4%. [b]1 G = 10^{-4}T. [c]The species distribution presented here is obtained from the analysis of the EPR spectra.

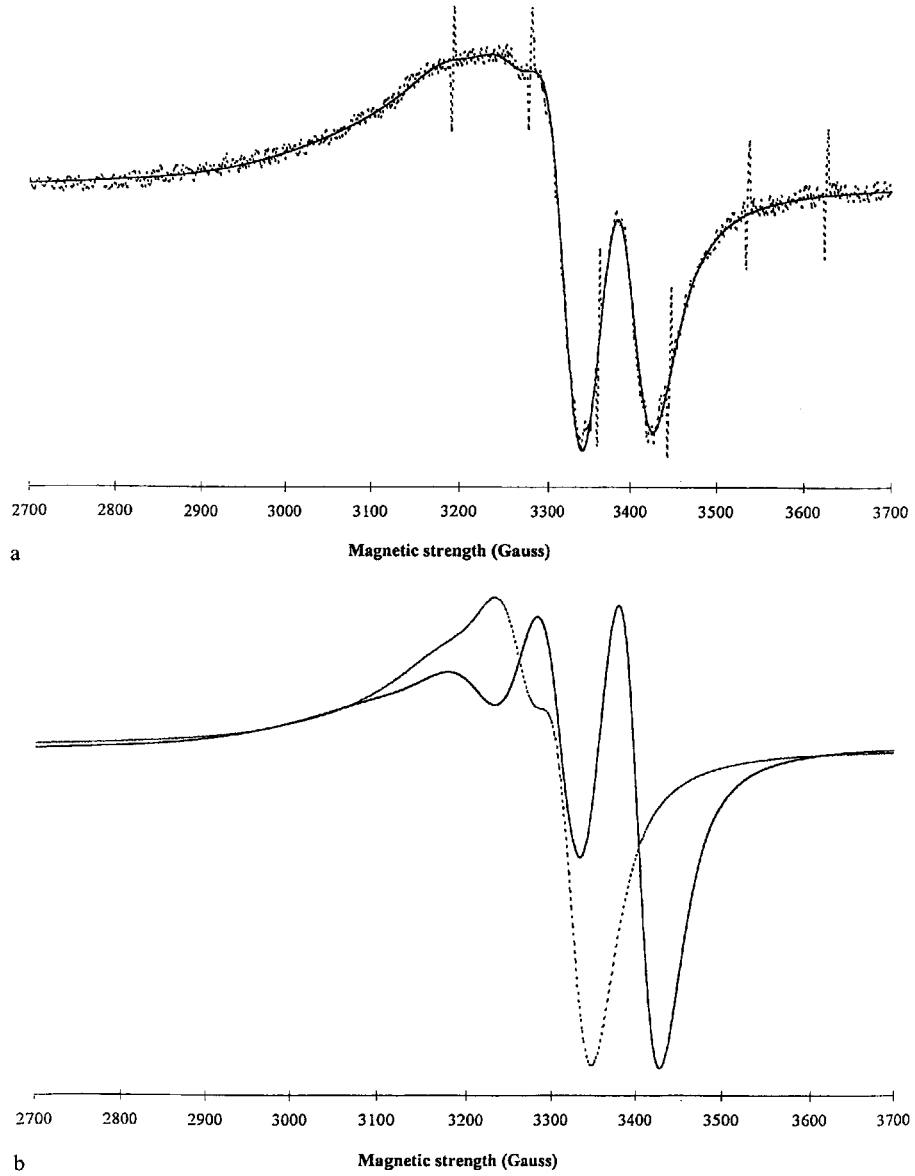

Fig. 4. The analysis of the EPR spectrum at pH 6.9 (L:M = 2:1, $C_{Cu^{2+}} = 5 \times 10^{-3}$ M). (a) The dashed line represents the experimental spectrum together with the external manganese field standard, while the solid line is the calculated curve. (b) The spectra (dashed line stands for $[ML]^{+}$ and solid line for MLH_{-1}) of the pure species contributing to the fitted one in 49:51 ratio, respectively.

systems, indicating that the two copper(II) centers in the binuclear complexes are not close to each other, i.e. there is no strong antiferromagnetic interaction between them which would result in spin pairing, as, e.g., in the hydroxo-bridged complexes,[31,32] and diminish the EPR spectra. The spectra are similar to those of the dimeric copper(II) complexes, where the ligands serve as bridges providing relatively good separation of the metal ion centers.[25,29,32] Further investigations are, however, needed to describe in more detail the structure of these binuclear complexes.

Conclusions

The investigation of copper(II) complexes of hexapeptide L-Lys-L-Leu-L-Ala-L-His-L-Phe-Gly by combined potentiometry, spectrophotometry, CD and EPR spectroscopy once more confirmed that these methods give useful complementary information in aqueous solutions. To our knowledge this is the first case in which all these methods were used under similar conditions for the examination of metal complexes of such a relatively large oligopeptide molecule. This is justified by, for example, the reasons described by Basosi[34] concerning the uncertainty of the frozen-solution EPR measurements.

The equilibrium study of a model system for the ACTH active site demonstrated that in different pH regions more than one metal complexes exist, which is usual for biologically active molecules. Therefore the knowledge of the species distribution is indispensable in order to determine the solution structure of the coordina-

tion compounds, as well as the quality and the arrangement of the donor atoms around the metal ion. On the basis of such investigations with a reasonable series of ligands one may come closer to the structure–activity relationships.

The results presented in this work show that the metal ion complexation changes the structure of the hexapeptide, which is the most important feature concerning its biological function. Until the physiological pH, complexes with a macrochelate ring (including the *N*-terminal α-amine and the imidazole nitrogen of histidine) and increasing number of deprotonated amide nitrogens are dominant. The imidazole nitrogen leaves the coordination sphere of the metal ion only above ca. pH 8, allowing the formation of classical peptide type coordination with fused five-membered chelate rings starting from the *N*-terminal α-amine up to the third deprotonated amide group, as in the case of peptides with non-coordinating side chains. Irrespective of the ligand excess applied during the measurements, only mono complexes were found in different protonation states. Binuclear complexes were detected, however, in the system with ligand to metal 1 : 2 concentration ratio with relatively separated copper(II) centers.

Further investigations are planned with hexapeptides of slightly different amino acid sequences and their cyclic analogues to obtaine more information about copper(II) binding properties of such ligands.

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References

1. Kiss, T. and Sóvágó, I. In Burger, K., Ed. *Biocoordination Chemistry*, Chap. III and IV, Ellis Hoorwood, London 1990.
2. Sigel, H. and Martin, R. B. *Chem. Rev. 82* (1982) 385.
3. Solomon, E. I., Hare, J. W. and Gray, H. B. *Proc. Natl. Acad. Sci. USA 73* (1976) 1389.
4. Coleman, P. M., Freeman, H. C., Guss, J. M., Murata, M., Norris, V. A., Ramshaw J. A. and Ventakappa, M. P. *Nature (London) 272* (1978) 319.
5. Jasani, B., Kreil, G., Mackler, B. F. and Stanworth, D. R. *Biochem. J. 181* (1979) 623.
6. Lawrence, I. D., Warner, J. A., Cohan, V. L., Lichtenstein, L. M., Kagey-Sobotka, A., Vavrek, R. J., Stewart, J. M. and Proud, D. *Biochem Pharmacol 38* (1989) 227.
7. Porunkevich, J. A., Ratkevich, M. P., Kublis, G. G., Mutulis, F. K. and Chipens, G. I. *Biohimija (Russ.) 55* (1990) 988; Ratkevich, M. P., Porunkevich, J. A., Ancans, J. E. and Chipens, G. I. *Biohimija (Russ.) 54* (1989) 1611.
8. Pickart, L. and Thaler, M. M. *J. Cell. Physiol. 102* (1980) 129; Daignault, S. A., Arnold, A. P., Isab, A. A. and Rabenstein, D. L. *Inorg. Chem. 24* (1985) 3984.
9. Gajda, T., Henry, B. and Delpuech J.-J. *Inorg. Chem. 34* (1995) 2455; Bal, W., Kozlowski, H., Robbins, R. and Pettit, L. D. *Inorg. Chim. Acta 231* (1995) 7.
10. Meldal, M., Bisgaard, Ch. H., Bojesen, G., Havsteen, M. J. and Holm, A. *Int. J. Peptide Protein Res. 41* (1993) 250.
11. Gausepohl, H., Pieles, U. and Frank, R. W. In Smith, J. A. and Rivier, J. E. Eds. *Peptides: Chemistry and Biology*, pp. 523–524, ESCOM, Leiden, 1991.
12. Zékány, L., Nagypál, I. and Peintler, G. *PSEQUAD for Chemical Equilibria*, Technical Sofware Distributors, Baltimore, MD, 1991.
13. Pettit, L. D., Pyburn, S., Bal, W., Kozlowski, H. and Bataille M. *J. Chem. Soc., Dalton Trans.* (1990) 3565.
14. Brookes, G. and Pettit, L. D. *J. Chem. Soc., Dalton Trans.*, (1975) 2112.
15. Rainer, M. J. A. and Rode, B. M. *Inorg. Chim. Acta 92* (1984) 1.
16. Lau, S-J. and Sarkar, B. *Biochem J. 199* (1981) 649.
17. Pickart, L., Freedman, J. H., Peisach, W. J., Perkins, C. M., Steinkamp, R. E. and Weinstein, B. *Nature (London) 288* (1980) 715.
18. Billo, E. J. *Inorg. Nucl. Chem. Lett. 10* (1974) 613.
19. Decock-Le Reverend, B., Liman, F., Livera, C., Pettit, L. D., Pyburn, S. and Kozlowski, H. *J. Chem. Soc., Dalton Trans.* (1988) 887.
20. Gajda, T., Henry, B. and Delpuech, J.-J. *J. Chem. Soc., Dalton Trans* (1993), 1301; Gajda, T., Henry, B. and Delpuech, J.-J. *J. Chem. Soc., Dalton Trans.* (1992) 2313.
21. Pettit, L. D., Steel, I., Formiczka-Kozlowska, G., Tatarowski, T. and Bataille M. *J. Chem. Soc., Dalton Trans.* (1985) 535.
22. Gyurcsik, B., Gajda, T., Nagy, L. and Burger, K. *J. Chem Soc., Dalton Trans.* (1992) 2787.
23. Koltun, W. L., Fried, M. and Gurd, F. R. N. *J. Am. Chem. Soc. 82* (1960) 233.
24. Jensen, H. P. and Larsen, E. *Acta Chem. Scand. 25* (1971) 1439.
25. Formicka-Kozlowska, G., Bezer, M. and Pettit, L. D. *J. Inorg. Biochem. 18* (1983) 335.
26. Livera, C. E., Pettit, L. D., Bataille, M., Perly, B., Kozłowski, H. and Radomska, B. *J. Chem. Soc., Dalton Trans.* (1987) 661.
27. Rockenbauer, A. and Korecz, L., Jr. *To be published.*
28. Wilson, R. and Kivelson, D. *J. Chem. Phys. 44* (1966) 154.
29. Rainer, M. J. A. and Rode, B. M. *Inorg. Chim. Acta 107* (1985) 127.
30. Szabó-Plánka, T., Peintler, G., Rockenbauer, A., Gyôr, M., Varga-Fábián, M., Institórisz, L. and Balázspiri, L. *J. Chem. Soc., Dalton Trans.* (1989) 1925.
31. Nagy, L., Yamaguchi, T., Nomura, M., Páli, T. and Othaki, H. *J. Inorg. Biochem. Submitted.*
32. Nagy, L., Yamaguchi, T., Mitsunaga, T., Wakita, H., Jezowska-Bojczuk M. and Kozlowski, H. *J. Chem. Soc., Perkin Trans. 2. Accepted for publication.*
33. Brown, Ch. E., Antholine, W. E. and Froncisz, W. *J. Chem. Soc., Dalton Trans.* (1980) 590.
34. Basosi, R. *J. Phys. Chem. 92* (1988) 992.

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