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[5-Leucine]- and [5-Methionine]enkephalin-Copper(II) Complexation under Physiological Conditions

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Paramagnetic perturbations in ^{13}C and ^{1}H NMR spectra of [Leu^5]- and [Met^5]enkephalins are caused by copper(II) ions and interpreted in terms of the major and minor species formed as a function of pH. The shifts in the electronic absorption spectra and the bonding parameters determined by EPR spectroscopy point to the formation of strong, covalently bonded species as the pH increases. The major species found under physiological conditions is described as involving the tyrosine amino group, two glycine peptide linkages, and the carboxylate function of the C-terminal residue. NMR broadening results are attributed to minor species involving axially bonded functional groups in rapid exchange. The significance of enkephalin-metal complexation lies in its possible use in determining the structures of the biologically active conformations.

Introduction

Since the discovery in brain tissues of the two pentapeptides Tyr-Gly-Gly-Phe-Leu and Try-Gly-Gly-Phe-Met by Hugues et al. in 1975,[2] interest in the properties of these natural enkephalins (Figure 1), and in related peptides, has increased dramatically. Research into the pharmacological properties of enkephalins has shown them to be potent analgesics[3] that operate at the same receptors as natural opiates and their antagonists.[4] Structure-function relationships have been investigated both theoretically and experimentally.[5]

The solution conformational states of the enkephalins have been intensively studied by nuclear magnetic resonance.[6-10] Initially, contradictory results were obtained, which were then resolved by admitting several sets of stable conformations featuring various types of β bends and different rotamer populations. The lability of the side-chain conformations of Tyr^1, Phe^4, and Met^5 residues remains a debated question.

Recent relaxation rate measurements focused on the structural information which can be derived from relaxation data dominated by dipolar mechanisms[6] in the presence of various solvents[7] and to possible biological binding components.[11] Lanthanum and gadolinium have been used to relate solvent polarity with the fraction of folded conformations and the separation of the charged end groups.[12] K^+, Na^+, Ca^{2+}, and Mn^{2+} have also been used to study alterations in the circular dichroism spectra,[13] but metal ion complexation has not yet been reported for the enkephalins.

Though no involvement of enkephalins in interactions with metal ions in vivo is known up to date, complex formation with metal ions seems to promise interesting results.[14] Several studies with model peptides have shown them to be strong complexing agents,[15] and one can in effect consider bioorganic compounds as competing for the various trace elements available. Even though much is known about the formation of copper complexes with the amino acids, this information cannot be easily transposed to the case of peptides, where detailed coordination studies have to be carried out in each case.

The dispersity of the theoretical results and the divergence of interpretations of the experimental results concerning the solution structure of enkephalins calls for the application of additional techniques. Since NMR line-broadening data have been used and questioned repeatedly,[16] we have used ^{1}H and ^{13}C NMR together with EPR and visible spectroscopy results. We now wish to report on the pH-dependent interactions of copper(II) with natural enkephalins in aqueous solution simulating physiological serum media.

Experimental Section

Synthesis. [Leu^5]enkephalin and [Me^5]enkephalin were both synthesized by solution methods. The ^{1}H and ^{13}C spectra are similar to published ones, with no spurious absorptions.

Instrumentation. Fresh D_2O solutions, 0.9×10^{-2} M in NaCl and 0.08-0.10 M in enkephalins, were used for NMR. DCl and NaOH solutions were used to adjust the pH which was measured with Tacusel 268 N and Orion Research pH meters and a Markson microelectrode. pH values are not corrected for deuterium isotope effects.

Solutions with variable copper concentrations were made up by micropipetting from a concentrated D_2O solution of the anhydrous copper chloride. Spectra were recorded at ambient probe temperature (300 K), and the shifts $\delta(C_i)$, $\delta(H_i)$ are expressed in ppm relative to external Me_4Si. Bruker WH90 and WH 400 NMR spectrometers operating in the Fourier transform mode were used for ^{13}C and ^{1}H spectra, respectively. The number of scans varied from 10000 to 25000 for ^{13}C and from 40 to 100 for ^{1}H spectra. Continuous-wave H_2O spectra were obtained on a Varian A-60 instrument, with a coaxial sample tube containing acetone as a line-width standard. A 10^{-3} M Cu^{2+} solution containing a tenfold excess of ligand was repeatedly scanned at 50 Hz width, resulting in an accuracy of better than 0.1 Hz.

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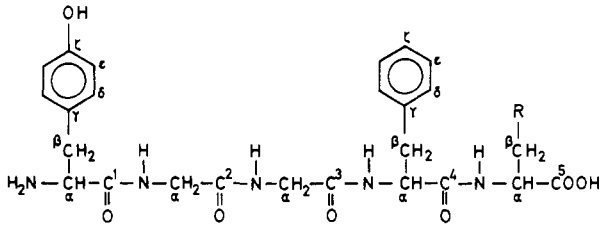

Figure 1. Primary structure of natural enkephalins. In [Met^5]-enkephalin, R is CH_2SCH_3; in [Leu^5]enkephalin, R is $CH(CH_3)_2$.

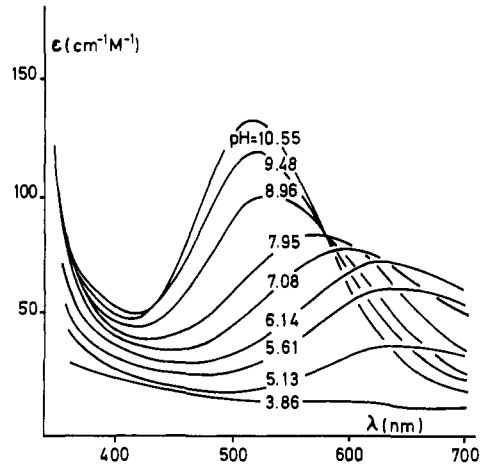

Figure 2. Absorption spectra of copper(II) [Leu^5]enkephalin complexes.

Solutions of 2×10^{-3} M Cu^{2+} in a 1-cm length cell and a Cary 14 spectrophotometer were used for visible absorption studies. An E-9 Varian EPR spectrometer was used with quartz sample tubes of various sizes, and the low-temperature spectra were recorded as previously described.[17]

Theory

Interpretation of the magnetic resonance results in the presence of Cu^{2+} ion is not straightforward and requires some theoretical background. NMR line widths are determined by the spin–lattice relaxation time $1/T_1$ and the spin–spin relaxation time $1/T_2 = \pi(\Delta\nu_{1/2})$ where $\Delta\nu_{1/2}$ is the width of the absorption at half-height. The presence of a paramagnetic metal ion can greatly change the parameters describing the NMR absorption, both by direct dipole–dipole interaction between the nuclear spin and the electron spin and by a contact interaction which transfers electron spin density from the metal ion to the nucleus being observed.[18,19] Since copper(II) ions have long electronic relaxation times ($T_{1e} = 2 \times 10^{-8}$ s) and the complexes fast rotational rates,[20] the contribution of the electronic spin to the nuclear spin relaxation is given by eq 1 where r is the distance between the two spin centers, τ_c the

$$\frac{1}{T_{2m}} \propto \frac{\tau_c}{r^6}\langle\mu^2\rangle + A^2\tau_c\langle\mu^2\rangle \qquad (1)$$

correlation time for the dipolar interaction, μ the magnetic moment of the paramagnetic ion, and A the hyperfine coupling constant.

Results

Visible Absorption Spectra. Formation of the different copper(II) enkephalin complexes with increasing pH can be followed by visible spectroscopy in the 350–700-nm range. Copper(II) [Leu^5]enkephalin was titrated from pH 3.5 to 12.5 and copper(II) [Met^5]enkephalin from pH 5 to 11. Absorption spectra were taken at various points during the titration. At low pH (<5) the absorption spectrum of copper(II) [Leu^5]-enkephalin presents a broad maximum near the infrared region with a very small molar absorbance (Figure 2). As the pH increases above pH 5, absorption maxima can be determined easily, as they progressively shift into the visible region, with concomitant increase in extinction coefficient.

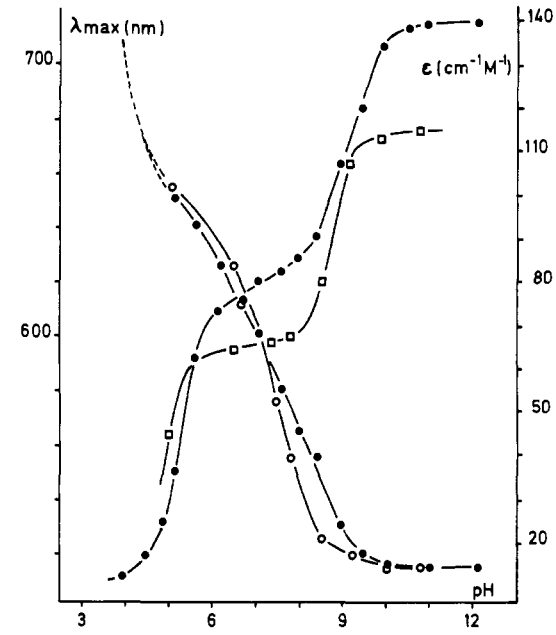

Figure 3. pH dependences of the wavelength maxima and the molar absorbance of copper(II) [Leu^5]enkephalin (full points) and copper(II) [Met^5]enkephalin (open symbols). Below pH 4.5 λ_{max} is not well defined in the 700-nm region and the ϵ values are calculated for a λ_{max} = 700 nm.

Copper complex formation can be detected visually as the solution changes from a colorless to pale blue color at low pH values and then to sea blue in the pH range 5–8.5. The solution then turns to a delightful violet color under increasingly basic conditions. Figure 3 shows a plot of the wavelength of maximum absorbance as a function of pH. The variations in extinction coefficients are also plotted in Figure 3 and reveal stepwise variations caused by changes in the ligands entering the copper coordination sphere. It may be noted that near neutral pH values the crystal field strength is increasing whereas the molar absorption reaches a plateau.

EPR Spectra. Electron paramagnetic resonance spectra of enkephalin solutions containing cupric ions show clear evidence of paramagnetic complex formation. Figure 4a,b shows the pH variations in spectral features for [Leu^5]enkephalin; similar spectra are obtained with [Met^5]enkephalin. A gradual change in the number of absorption lines appears simultaneously with a shift of the overall spectrum to higher field strengths, as the pH increases. At intermediate pH values, several complexes coexist in solution. Frozen-solution spectra reveal the same general pattern, with good resolution of the components, and with additional structure, which can be attributed to nitrogen ligation under basic conditions.

A major difference in the appearance of the liquid-solution spectra is seen when an excess of enkephalin is used (see Figure 4b). Formation of complexes at somewhat earlier pH values, for example, pH 7.3 instead of 7.5, is related to a small displacement of the equilibria toward complex formation. But the most striking feature is the loss in resolution near pH 7. Formation of copper hydroxyde can be ruled out because of

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Table I. Spectroscopic Data and Bonding Parameters for Copper(II) [Leu^5]enkephalin Complexes

pH	$\nu_\parallel$, cm^{-1}	$10^{-4} \times A_\parallel$, cm^{-1}	$g_\parallel$	$g_\perp$	$10^{-4} \times A_0$, cm^{-1}	g_0	k_0^2	α^2	γ^2
3.2	13500	165.1	2.358	2.073		2.194	0.81	0.88	0.92
4.5	14300	159.7	2.280	2.061	67.5	2.189	0.81	0.79	1.03
5.9	15600	176.5	2.224	2.055	65.7	2.131	0.60	0.78	0.77
7.5	17400	176.5	2.224	2.051	81.7	2.108	0.56	0.77	0.72
8.7	19050	194.8	2.196	2.051	98.0	2.099	0.56	0.79	0.70
9.3	19200	199.3	2.189	2.051	98.9	2.098	0.55	0.80	0.69
11.2	19400	198.6	2.182	2.051	97.7	2.093	0.53	0.79	0.67

the excess of ligand, but polynuclear complexes can still be considered. We prefer, on the basis of supporting NMR evidence, to interpret this result as indicating rapid exchange between the major copper enkephalin complex and various ligand nuclei occupying momentarily the axial coordination sites.

Metal–Ligand Bonding. The in-plane σ-bonding coefficients can be calculated the usual way.[21] The in-plane σ-bonding covalency parameter α^2 is given in Table I. The α^2 values diminish from 0.88 to 0.77 as complex formation takes place and then stabilize around 0.79. On the whole, the parameters are reasonable when compared with other complexes.[22,23]

A well-known correlation exists between the values $A_\parallel$, and $g_\parallel$ and bond strenghts in copper complexes. This has been widely used to discuss covalency in protein compounds.[24] Thus, the observed diminishing $g_\parallel$ values are in agreement with the strengthening of the copper–enkephalin bonds.

The usual approach is to assume D_{4h} symmetry with σ bonding between the copper $d_{x^2-y^2}$ orbital and the A_{1g} orbitals of sp^2-hybridized nitrogens. Then, the anisotropic g values will yield in-plane σ- and π-bonding parameters through the usual relations[25] existing between the g tensor elements and the crystal field splittings. However, various approximations have been used with room-temperature optical spectra to obtain the needed energy separations.[26]

We calculated a mean bonding coefficient k_0^2 from the appropriate expression for the isotropic g_0 value, eq 2.

$$g_0 = g_e - \frac{4\lambda k_0^2}{\Delta E} \qquad (2)$$

Reliable k_0^2 values can be derived from the λ_{max} data observed and a spin–orbit coupling constant of -829 cm^{-1}. k_0^2 is also termed the orbital reduction factor.[27] The values obtained show significant deviations from the α^2 values also presented in Table I. Setting $k_0^2 = \alpha^2\beta^2$ or $k_0^2 = \alpha^2\gamma^2$, one can use the results to calculate a secondary bonding parameter. For complexes in acidic solutions, no π bonding can occur with saturated amino groups, and only weak π bonding could contribute to the copper carboxylate or protonated peptide group. This is consistent with the large secondary binding parameters obtained, close to 1. One can therefore set $k_0^2 = \alpha^2\beta^2 \approx \alpha^2$. Deprotonated peptide groups, on the other hand, have suitably oriented π^* orbitals and back-bonding from the d_{xz} or d_{yz} electrons to the peptide π^* orbitals is indicated.[28]

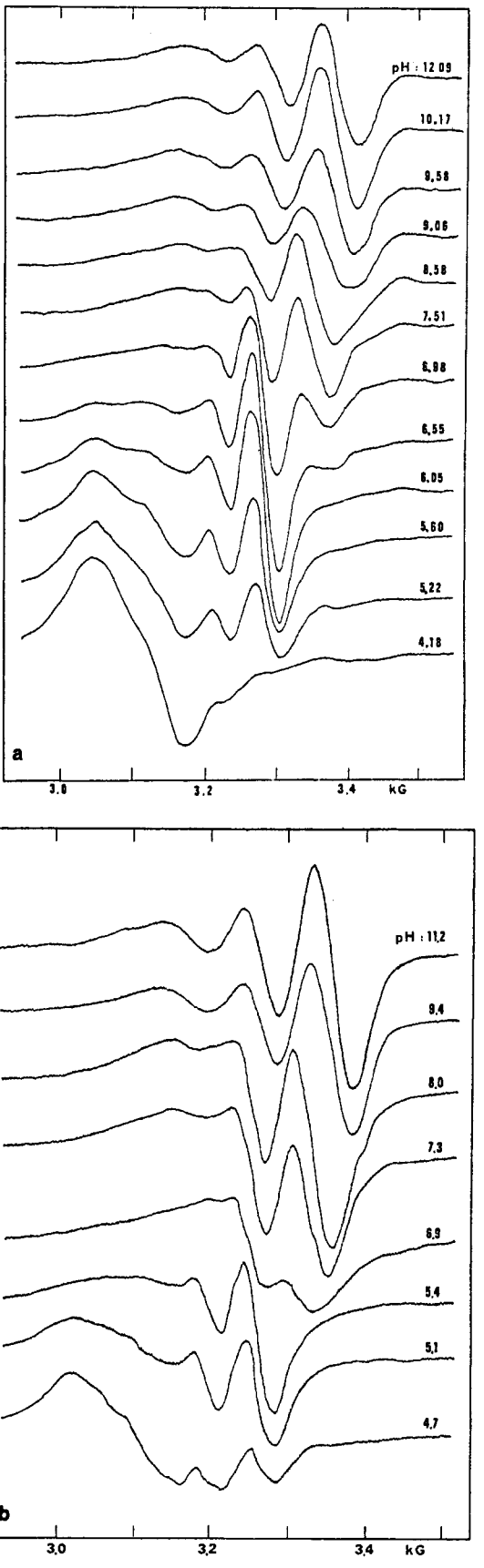

Figure 4. (a) pH dependence of EPR spectra of 1:1 copper(II)–[Leu^5]enkephalin solutions at room temperature. (b) pH dependence of EPR spectra of 100:1 copper(II)–[Leu^5]enkephalin solution at room temperature.

The out of plane π-bonding parameters calculated as $\gamma_0^2 = k_0^2/\alpha^2$ are listed in Table I. Considering the low values of

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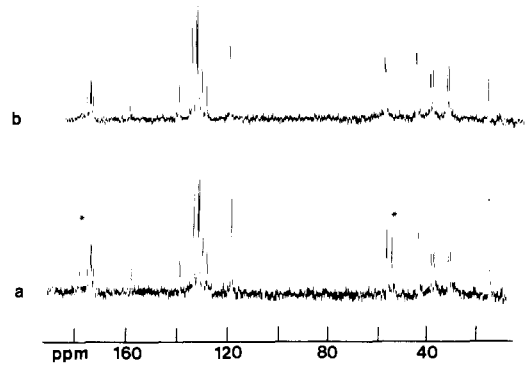

Figure 5. Spectra of the cationic form of [Me^5]enkephalin at pH 3.3 (a) without and (b) with copper. Peaks near 170 ppm are carbonyl carbons. Those near 130 ppm are aromatic carbons, and those around 40 ppm are aliphatic carbons. Complete assignments are found in the literature (see Results). In Figures 5–7 spectra in presence of copper(II) were obtained with a ratio $[Cu^{2+}]/[ligand] = 10^{-2}$. The peaks affected by addition of Cu(II) are indicated with asterisks.

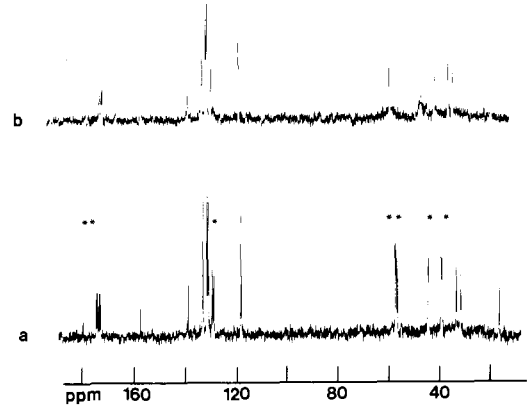

Figure 6. Spectra of the dipolar form of [Met^5]enkephalin at pH 7.4 (a) without and (b) with copper.

α^2 and γ^2 for the copper [Leu^5]enkephalin complex obtained at high pH, one must admit the formation of strong σ and π bonds between the metal ions and the peptide binding groups. Within experimental error, [Met^5]enkephalin gave identical results, suggesting the sulfur atom does not participate in copper binding.

NMR Spectra. The ^{13}C resonances were assigned with the help of previously published results.[10,29]

Addition of cupric ions to solutions of the enkephalins, in ratios of peptide to metal of 100:1, produces a significant change in ^{13}C spectral features. Figure 5 shows the effect of copper on the cationic form of [Met^5]enkephalin. Whereas no chemical shift differences can be detected within experimental error, two peaks are clearly missing, corresponding to the Met^5-carboxyl and Met^5-α carbons. The dipolar form of the peptide is also severly perturbed, as shown in Figure 6. In this case, most peaks are broadened, and several become undetectable. In addition to the Met^5-carboxyl and Met^5-α carbons, the Tyr^1-carbonyl and Tyr^1-α carbons have disappeared. The α-carbons of Gly^2 and Gly^3 residues are greatly affected, as well as the other tyrosine carbons in a progressive fashion. Whereas the Tyr β and Tyr γ are barely visible, the Tyr δ and Tyr ζ are reduced in intensity and the Tyr ϵ slightly less so.

The anionic form of [Met^5]enkephalin is yet differently affected by cupric ions, as shown in Figure 7. The Met^5 and

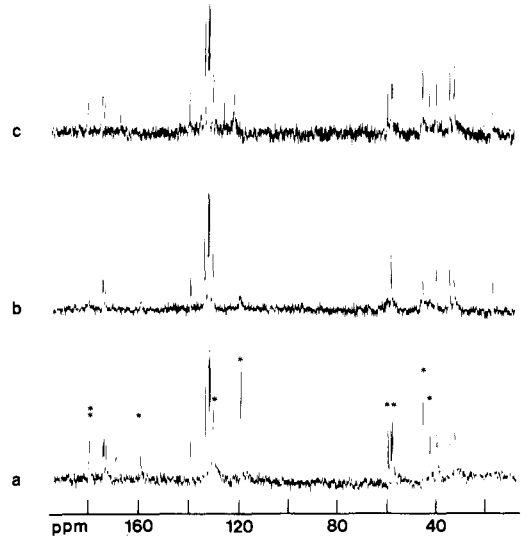

Figure 7. Spectra of the anionic form of [Met^5]enkephalin at pH 9.7 without copper (a) and with copper at pH 9.7 (b) and pH 10.8 (c).

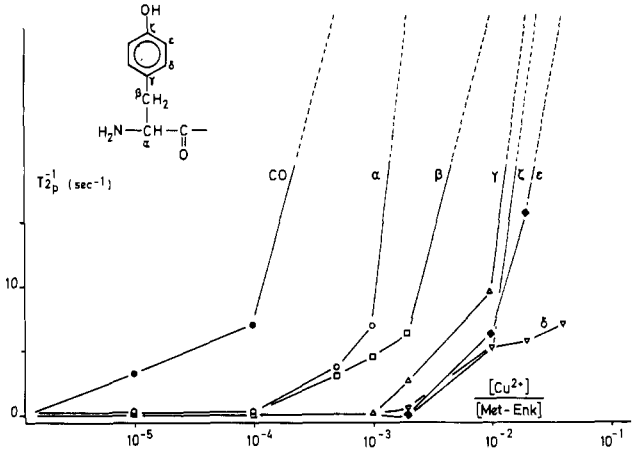

Figure 8. T_{2p}^{-1} dependence of the ^{13}C resonances of the tyrosyl residue on the molar ratio $[Cu^{II}]/[[Met]enkephalin]$ at pH 8.3.

Tyr^1 carbonyl, Met^5-α, Tyr^1-α, as well as the Tyr^5-β carbons have broadened beyond detection. One (or both) of the Gly^2 and Gly^3 α-carbons is affected. The Tyr δ carbon is least affected, whereas the Tyr ζ and Tyr ϵ carbons are now strongly broadened. These same progressive changes are also seen in [Leu^5]enkephalin spectra, as well as in all other spectra at other pHs. At pH 10.8, a noteworthy transformation takes place, all the absorptions being restored to intensities and line widths approximately equal to the metal-free case.

So that selective effects in paramagnetic broadening could be checked, line widths were investigated as a function of cupric ion concentration. Unchanged spectra are obtained when the copper to ligand ratio is 10^{-6} or less. If this same ratio exceeds 4×10^{-2}, paramagnetic broadening is too severe to yield any worthwhile NMR spectra. Figure 8 shows how the [Met^5]enkephalin peptide ^{13}C transverse relaxation times vary as the copper concentration increases at pH 8.3. Nonlinear variations are also obtained for [Leu^5]enkephalin. At pH 8.3, paramagnetic perturbations yield important clues on copper binding to the tyrosine residues. However, the Gly^2-α and Gly^3-α carbons are not resolved at this particular pH value and their involvement in metal binding cannot be determined unambiguously from ^{13}C data alone. Additional ^1H NMR spectra help in this respect. Figure 9 shows how the amide region is affected by copper introduction into a Me_2SO solution of the ligand. With previous assignments derived from double resonance techniques, the most affected adsorption is deter-

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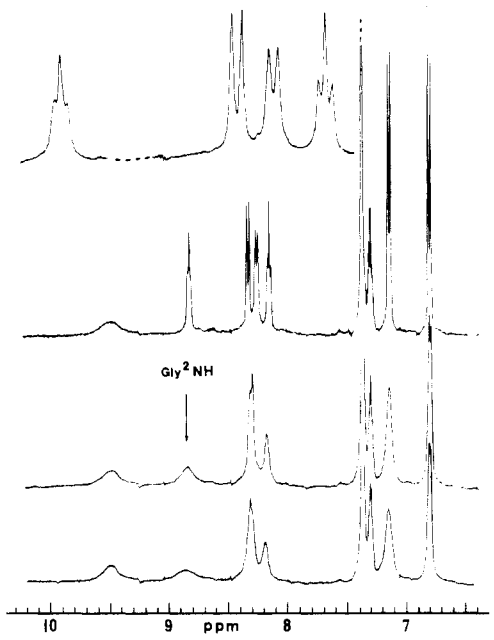

Figure 9. Lower field region of the 400-MHz ^1H NMR spectra of [Leu^5]enkephalin in Me_2SO solution with increasing amounts of copper(II) ion (from top to bottom). Upper insert represents expanded low-field region.

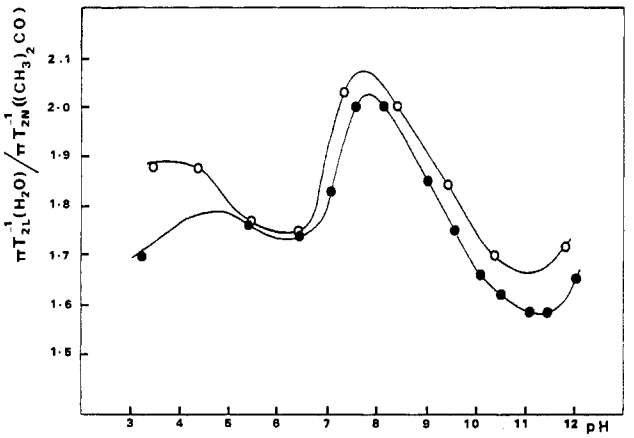

Figure 10. Proton-relaxation enhancement of solvent H_2O in the presence of copper(II)–enkephalin complexes (open symbols represent copper(II) [Met^5]enkephalin and filled ones copper(II) [Leu^5]enkephalin).

mined as the Gly^2 amide. Other spectra run in D_2O show extensive broadening of the tyrosine ring protons, whereas the Phe^4, Leu^5, and Met^5 protons are the least affected. Under highly acidic (pH 2.35) or basic (pH 11.2) conditions, the copper-free spectra are restored.

The broadening of the H_2O solvent peak is also slightly pH dependent, but the line width does not increase beyond 1.5 Hz for a 10^{-3} M Cu^{2+} solution (Figure 10).

Discussion

Much effort is devoted to studying the structure of peptidic metalloenzymes containing copper[30,31] as well as simpler amino acid complexes.[32] Most studies of the interactions of copper(II) with small peptides have been successfully interpreted on the basis of square-planar complex formation.

Electronic Absorption Spectra. According to the classical ligand field theory, as stronger ligands enter the copper coordination sphere and replace water molecules, the crystal field strength increases and the absorption shifts to higher energies. Thus $\lambda_{max} > 700$ nm corresponds to a copper complex bound by oxygen atoms whereas the blue color of amino acid complexes with $\lambda_{max} \simeq 630$ nm is typical of square-planar coordination by two oxygen and two nitrogen donor ligands.[33,34] The color change to violet under alkaline conditions is not observed with simple copper amino acid complexes, but this color is the observed one in biuret or glycinamide complexes and has been observed before in copper peptide complexation.[35,36]

With the unpaired electron in the $d_{x^2-y^2}$ copper orbital, the symmetry-forbidden transitions responsible for the weak absorptions (the extinction coefficients are less than 100 cm^{-1} M^{-1}) involve the ^2E_g ground state and $^2T_{2g}$ excited state, which are futher split by tetragonal geometry. In some cases the broad absorption bands have been resolved into various components,[37] but the main absorption[38] is $^2E_g \leftarrow {}^2B_{1g}$. The deprotonated peptide linkage contains a planar π system, and the bound nitrogens have unoccupied π orbitals of e_g symmetry. These can overlap with the metal d_{xz} and d_{yz} orbitals, also e_g, resulting in a strengthening of the Cu–N bond by π backbonding.[28] The lowering of metal e_g orbitals would appreciably increase the energy of the main electronic transition, in agreement with the blue-shift observed experimentally. The larger extinction coefficient is also consistent with admixture of ligand character in the metal e_g orbitals. This is also verified by the EPR results.

With use of the formulation of Billo,[33] the absorption energies can be calculated as ν_{obsd} (10^3 cm^{-1}) = $4.85n$ (peptide) + $4.53n$ (amino) + $3.42n$ (carboxylate) + 3.01 (H_2O, protonated peptide). With one amino, carboxylate, water, and protonated peptide in the copper coordination sphere, the calculated λ_{max} is 715 nm, comparing favorably with the observed values under acidic conditions. One amino, one carboxylate, and two peptide groups yield a calculated λ_{max} = 567 nm, which is observed near pH 8. Finally, one amino and three peptide groups give λ_{max} = 524 nm, a value reached experimentally near pH 9, strongly suggesting deprotonated peptide groups are binding copper under alkaline conditions.

EPR Parameters. The results obtained for [Met^5]- and [Leu^5]enkephalin are similar in most respects, except that copper [Met^5]enkephalin solutions tended to decompose on standing, producing additional EPR absorptions near $g = 2$. These can be attributed to organic decomposition materials of the methionine end group.

The observed variations in the liquid-solution spectra as a function of pH are typical of copper peptide systems. Under acidic conditions, a single broad absorption results from the rapid tumbling of the relatively small hydrated cupric ions. Coordination of labile carboxylates or protonated peptide groups is not expected to greatly change the broad absorption line.

Above pH 4, however, when a stronger chelate-like complex begins to form, additional absorptions appear at higher fields (see Figure 4). As the equilibrium is shifted toward chelate

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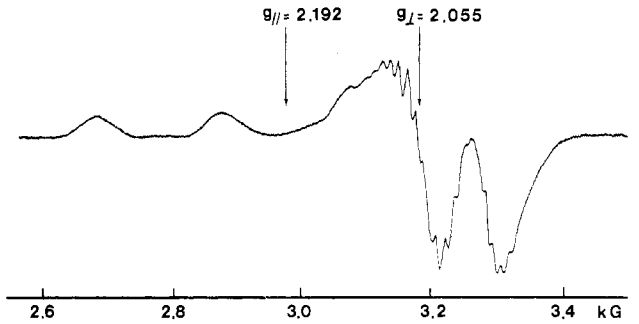

Figure 11. 77 K frozen-solution spectrum of copper(II) [Leu^5]-enkephalin at pH 9.2, showing resolved hyperfine structure.

formation, the four-line pattern of copper complexes becomes resolved. Thus, at pH 6.5, the observed spectrum is almost entirely due to a single species with $g = 2.131$ and an isotropic hyperfine coupling constant of about 65 G. The first derivative of the absorption spectra (as presented in Figure 4) appears unsymmetrical because of the dependence of the relaxation times on the nuclear magnetic moment quantum numbers.[39] The narrowest line of peak to peak width (40 G) corresponds to a Lorentzian line width of $\Gamma = 34.6$ G. This gives $T_2 = (\gamma 2\Gamma)^{-1} = 5 \times 10^{-9}$ s. Considering the normal spin–lattice relaxation time for copper, $T_1 \approx 2 \times 10^{-8}$ s, one can see from the relationship $T_2^{-1} = T_{2e}^{-1} + 0.5T_1^{-1}$ that the line widths are governed by the electronic spin–spin relaxation times T_{2e}.[40]

Starting at pH 6.5, a new compound appears as a shoulder on the high-field side of the previous absorption. This becomes the main spectrum in the interval $7.5 < \text{pH} < 8.5$. Its hyperfine coupling constant is larger, $A_0 = 86$ G, and g value smaller, $g_0 = 2.108$. This spectrum is in its turn replaced by another of yet different spectral parameters, $A_0 = 100$ G and $g_0 = 2.098$. At pH 12, the peak to peak width is slightly larger, 50 G, indicating that T_{2e} has possibly diminished. But, as the four-line copper hyperfine structure seems better resolved, we would rather attribute the small but significant increase in line width to a contribution from the spin–rotational relaxation mechanism which is independent of the nuclear magnetic moment quantum number. Under alkaline conditions, enkephalins can be considered as tightly bound around the copper nucleus, resulting in a rotational correlation time of about 2×10^{-11} s. This would give a calculated line width contribution of 3×10^8 s^{-1} at room temperature.[41] The rest of the line width can be assigned to unresolved ligand hyperfine structure. Strongly basic solutions can also yield copper hydroxy peptide complexes.[42]

Frozen solutions of the copper enkephalin complexes give resolved anisotropic spectra, as shown in Figure 11. These spectra are typical of tetragonal copper complexes with $g_\parallel = g_z$ and $g_\perp = g_x = g_y$; the data for [Leu^5]enkephalin is presented in Table I and explains why isotropic spectra only are observed for liquid solutions of copper enkephalins. So that the anisotropic spectra can be observed, the rotational rate must be smaller than $(g_\parallel - g_\perp)\beta H_0/h$, which is 1.3×10^8 s^{-1} in our case. Anisotropic liquid-solution spectra have been observed only in much larger copper enzyme systems.[24]

The nuclear hyperfine structure $A_\parallel$ is resolved on the $g_\parallel$ part of the spectrum and, in some cases, hyperfine structure also appears on the $g_\perp$ part. Some discrepancy exists in the interpretation of the lines found in the perpendicular region. That the lines in the perpendicular region are due to nitrogen ligand nuclei is not obvious in copper enzyme systems, and they have been assigned as copper hyperfine $A_\perp$.[43] However, in smaller peptide systems, isotropic nitrogen substitution has been observed to influence ligand hyperfine lines.[44] In any case, the copper EPR can be interpreted in terms of the usual tetragonal spin Hamiltonian used for square-planar complexes.

Ligand Hyperfine. Figure 11 shows the well-resolved nine-line hyperfine structure obtained for copper [Leu^5]-enkephalin at pH 9.2. The number of lines is compatible with coupling to four equivalent nitrogen nuclei. However, as previously mentioned, the coupling constant of 1.33×10^{-3} cm^{-1} is close to the calculated value of $A_\perp$ for copper.

Using eq 3, which describes the isotropic interaction energy

$$E_N = (4\pi/9)\gamma_n\beta_0\beta_N\alpha^2(\delta(r)) \qquad (3)$$

between electronic and ligand nuclear moments,[21] one can calculate the expected value of the nitrogen hyperfine coupling constant A_N. The usual s-spin density at nitrogen is taken as 33.4×10^{24} cm^{-3}, which implies sp^2 hybridization. Also α^2 is obtained through the normalization procedure $\alpha^2 + (\alpha')^2 \pm 2S\alpha\alpha' = 1$, where the overlap integral is taken as $S = 0.093$. With $\alpha^2 = 0.79$, $(\alpha')^2 = 0.30$ and the calculated splitting is about 14.5 G, slightly larger than the observed value (13.75 G). Some hyperfine structure is also visible at pH 8, but the number of lines does not coincide with any theoretical model, and the coupling constant is of doubtful value. Frequent observations[22] have been made concerning discrepancies in α^2 values calculated from eq 2 and 3. We feel the problem is related to the assumed α nature of the copper–peptide bond. Our results show the π bond ($\gamma^2 = 0.67$) is more "covalent" than the σ bond ($\alpha^2 = 0.79$).

NMR Spectra. Equation 1 has been the basis of many NMR line-broadening studies in which the $1/r^6$ dependence has been used to delineate the nuclei closest to the paramagnetic metal ion and therefore to determine the binding sites and overall geometry of the complex in solution.[45] However, when the complex undergoes chemical exchange or dissociation characterized by τ_m, the lifetime of the ligand bound to the metal ions, then the observed line width depends on P_m, the fraction of ligand bound to the metal, and Δw_m, the chemical shift difference between bound and free ligand.[46]

In our experiments, we detected no variation in the chemical shifts of the free ligands even for the largest metal to ligand ratios used. This is consistent with other observations on Cu(II) systems,[47] where relaxation was found to occur by a T_{2m} mechanism. Taking into account paramagnetic relaxation occurring in the second solvation shell ($1/T_{2os}$, for "outer sphere"), the observed line width is given by eq 4.

$$\frac{1}{T_{2obsd}} = \frac{1}{T_{2a}} + \frac{P_m}{T_{2m} + \tau_m} + \frac{P_m}{T_{2os}} \qquad (4)$$

In the case of "slow exchange", the lifetime of the bound ligand must be $\tau_m > T_{2m}$, which is usually estimated between 2×10^{-4} and 10^{-6} s for copper complexes.[48,49] Most NMR broadening studies rely on "fast exchange" conditions, so that the linewidth is controlled by T_{2m}, in eq 4, and structural information can then be derived from the r^{-6} dependence shown in eq 3. Authors argue that fast exchange is verified by decreasing line widths as the temperature rises. The inherent

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difficulty arises, however, from the fact that for a given ligand, as the distance from the metal increases, T_{2m} drops off sharply and eventually the "fast exchange" condition $\tau_m < T_{2m}$ may no longer be valid for the more distant nuclei.[50] Therefore, differential broadening of ligand nuclei may not simply correlate with the simple $1/r^6$ dependence of $1/T_{2m}$, as some nuclei may be in the fast-exchange region while others are in the slow-exchange one. Furthermore, recent results[49] have been interpreted in terms of equal contributions to the total observed line width from dipolar interactions (T_{2m}) and from "outer sphere" relaxation (T_{2os}). Observed line broadenings have also been attributed to predominantly scalar interactions,[51] implying the A^2 term is dominant in eq 1. Consequently, selective broadening experiments may not reflect the explicit r^{-6} distance dependence of the dipolar term, even though they may still be used to determine the sites of Cu(II) binding.

^{13}C Results. The ^{13}C line-broadening results in Figure 5 show that the C-terminal end of the peptide is first affected by copper at pH 3.3. This is the expected result if one proposes ionic bonding between Cu^{2+} and the deprotonated carboxylic acids of the enkephalins. The fraction of carboxylic acids which are protonated is in rapid equilibrium with the deprotonated ones, accounting for the overall broadening.

Both ends of the peptides are affected by copper ions under less acidic conditions (pH <6). This behavior can be compared to that observed previously with other peptides, where terminal amino groups were found to be broadened by copper before peptide and carboxylato end groups.[52] This was taken as evidence that terminal amino and peptide groups rather than carboxylato groups are the preferred binding sites. However, Espersen and Martin interpreted these results as caused by kinetic effects, terminal amino groups exchanging more rapidly in the presence of a 5000-fold excess of ligand, than the rate-determining chelate ring-closure step following copper-induced amide hydrogen ionization. Our data, on the other hand, clearly show broadening of the C-terminal [Met^5] and/or [Leu^5] groups followed by broadening of the N-terminal [Tyr^1] group as the pH rises. One of the glycine residues is also involved, as can be determined by broadening of either carbon or proton CH_2 resonances. The progressive disappearance of the [Gly^2] amide proton resonances in Me_2SO (Figure 9) shows that the peptide linkage next to the N-terminal Tyrosine is the first to be affected. Margerum found copper tripeptide complex formation[53] to proceed quite rapidly. ($K = 10^7$ M^{-2} s^{-1}). We conclude that the terminal NH_2, the first peptide linkage, and the terminal COO^- groups are bound in chelate-like fashion to the copper ions in slightly acidic solution.

At physiological pH, similar broadening results are obtained. The selective broadening pattern of the tyrosine residue changes under more alkaline conditions, as the phenol group becomes deprotonated. Whereas at pH 7.4 the aromatic alcohol seemed to be the furthest from the paramagnetic center, it is strongly broadened above pH 8.3 (see Figure 8). This implies, in our opinion, the presence of a rapidly exchanging species involving the copper-promoted deprotonated phenol group. The copper ions responsible for the broadening are rapidly exchanging between the phenolate anion and the amino amide part of tyrosine, thus explaining why the Tyr-δ carbon, half-way between the two copper-interaction sites, is least affected.

At still higher pH values, the line broadening of the ^{13}C resonances diminishes until the metal-free spectrum is restored starting at pH 10.8. As we found no evidence of copper precipitation as the hydroxide or reduction to diamagnetic colorless copper(I), the results must be interpreted in the following manner. At high pH, the copper(II) ions are tightly bound to the enkephalins, and very slow dissociation, or second-order ligand exchange, occurs. This is consistent with kinetic information on copper peptide or copper enzyme systems where the dissociation step was calculated to lie between 1 and 0.5 s^{-1}.[54] The observed NMR spectrum represents the excess unbound enkephalin which is not affected by the inert copper enkephalin complex. The line widths are given by eq 4, with "slow exchange" conditions. In dilute solutions, P_m is the ratio of the lifetimes in the paramagnetic and diamagnetic environments and is approximated by the respective concentrations: $(\tau_m/\tau_a) = [\text{complex}]/[\text{ligand}] \approx 0.01$ in our case. The outer-sphere relaxation rate is usually <100 s^{-1} [42,18] and the proportion of ligands in the outer coordination sphere of a copper peptide complex with no free axial binding site on the copper is not expected to be large (also 0.01) so that the total line width will be

$$\frac{1}{T_{2obsd}} = \pi(\Delta\nu_{1/2}) + \frac{1}{100 \times 1\ \text{s}} + \frac{1}{100 \times 10^{-2}\ \text{s}} = 10\ \text{s}^{-1} \approx \frac{1}{T_{2a}}$$

For completeness, we mention that the bound ligand relaxation is dominated by the large electronic magnetic moment of copper(II) and its long relaxation time, 2×10^{-8} s, which broadens the signal beyond detection.

^{1}H Results. The large paramagnetic broadening of the proton spectra of enkephalins observed in the presence of small amounts of copper at neutral pH indicates the metal ions are effectively exchanging between the various donor sites. At pH 11.2, no broadening is observed for the same reasons discussed for the ^{13}C results. At pH 2.35, the enkephalins are fully protonated and do not interact with cupric ions, explaining the absence of perturbations on the proton spectra.

Proton relaxation enhancements of solvent water molecules have been widely investigated as a means of following metal–ligand complex formation.[45] As shown in Figure 10 the H_2O line widths of copper enkephalin solutions are quite narrow and vary little as a function of pH when compared to other systems, including amino acids.[55] When an inner-sphere complex is formed with an organic ligand, the ligand replaces coordinated water and consequently reduces P_m in eq 4. Line broadening of the solvent is therefore expected to decrease as the solvent molecules are expelled from the paramagnetic coordination sphere. In agreement with recent work, we attribute the broadening at pH <7 to water molecules in fast exchange.[56] When the neutral copper complex is formed, broadening can still be attributed in part to rapidly exchanging solvent molecules coordinated in the axial positions. The lifetime of the water molecules ($\tau_m \approx 10^{-9}$ s) is even expected to decrease as axial solvent exchange proceeds more rapidly after neutralization of the charges on cupric ions by coordinated ligands. The slightly increased line broadening is mainly due, however, to rapid proton exchange between solvent and

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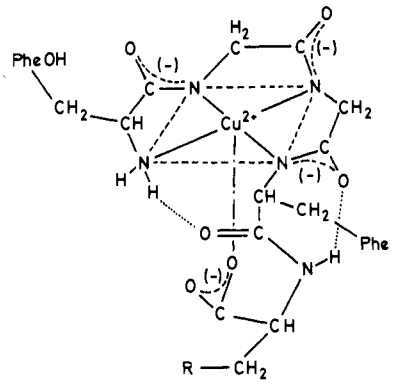
the coordinated ligand protons which are labile (amino and protonated peptide nitrogen). Second-order ligand exchange between copper and ligand followed by rapid proton exchange between ligand and solvent accounts for the line widths.[55] In basic media, the solvent broadening diminishes, as observed for the ligand ^{13}C resonances. If the enkephalins tightly occupy all the copper coordination sites and the peptide linkages are deprotonated, then no inner-sphere broadening mechanism remains. The residual line broadening is due to outer-sphere interactions.

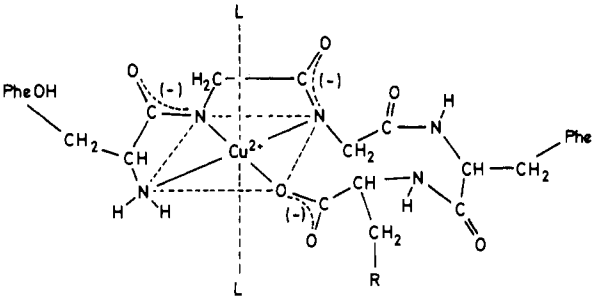
Proposed Structures. Basic Solutions. Structure I can be



I, $CuH_{-3}L^{2-}$

proposed for the stable copper enkephalin species found under alkaline conditions. The four nitrogens found in the preferred square-planar geometry around copper agree well with the observed EPR results. Furthermore, the structure allows for axial coordination of the terminal COO^- group and multiple hydrogen bonding of the unbound [Phe^4] amide function as proposed by Margerum in order to explain the CD spectra of copper pentapeptide[57] complexes. At lower pH values the crystal field strength around copper becomes weaker, and this is consistent with replacement of a deprotonated peptide–metal bond with a metal–carboxylate, –water, or –protonated peptide bond. Both general acid protonation and nucleophilic attack by excess ligand are expected to occur at the amide linkage between the [Gly^3] and [Phe^4] residues. Since the steric effects associated with glycine leaving groups are expected to be minimal,[15] dissociation of the copper–peptide bond could be rapid and protonation could determine the rate-limiting step. In such a case, "outside" protonation has been proposed, where the peptide oxygen adds a proton, with subsequent or concurrent metal–N(peptide) bond cleavage. A sluggish reaction of about 10^2 M^{-1} s^{-1} would explain why no broadening of the [Gly^3] is observed, as the calculated T_{2p}^{-1} value would be larger than 10^4 s. The NMR results still show, however, that the C-terminal enkephalin residues are affected, even at pH 9, strongly suggesting carboxylato groups remain in the coordination sphere of the metal ion.

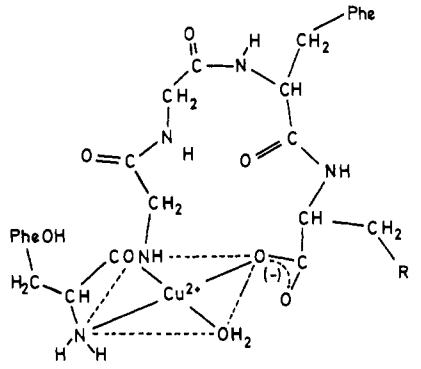
Neutral Solutions. Rapid dissociation of the entire complex is ruled out by the selective nonbroadening of one glycine and phenylalanine group. We propose that coordination around copper is square planar, as shown in structure II, with undetermined ligands in the axial bonding positions. These axial sites provide the labile coordination positions needed to explain the selective broadening results. It is logical to assume that the labile species preferentially involve metal binding sites of the peptides. Broadening is thus ascribed to "minor species" is fast exchange. In copper glycinate, for example, the lifetime of the third glycine molecule is 10^{-8} s so that in our case we can safely assume that $\tau_m < T_{2m}$ and apply 4 to the nuclei bound in the axial position. Using $\gamma_I^2 < \mu^2 > = 1.38 \times 10^{-44}$



II, $CuH_{-2}L^-$ (axial positions occupied by water or other rapidly exchanging ligand molecules)

m^6 s^{-2} [51] and τ_c controlled by $\tau_r = 10^{11}$ s, one obtains $T_{2m}^{-1} = 1.3 \times 10^3$ s^{-1} for a reasonable copper–proton distance of 3 Å. If this factor is equally distributed among four binding groups (carboxylato, phenol, peptide, and amino groups) then 3 Hz is contributed by P_m/T_{2m} to the proton line widths in axial positions. This value can be doubled by considering the expected scalar contribution[51] or the outer-sphere term.[49] Thus, the total line width (eq 4) will be $T_{2obsd}^{-1} = 13.2$ Hz, in good agreement with typically observed line widths. Structure II is compatible with the calculated and observed electronic spectrum and allows for multiple points of intramolecular interaction within the free oligopeptide chain portion or with the rest of the complex.

Acid Solutions. When the copper–enkephalin solutions are further acidified below physiological pH value, further dissociation of the complex takes place. From the EPR and visible spectra, copper complexation by water, one carboxylato group, and a terminal amino function is indicated. As proposed previously,[15] on the basis of potentiometric and kinetic results on smaller glycine containing peptides, the remaining two deprotonated peptide linkages should undergo simultaneous acidification and decomplexation, with a rate of the order of 10^3 s^{-1}. The final complex, shown as structure III, has been termed the "skipping over" complex, where in effect several potential binding sites are left untouched by copper (structure III).



III, CuL^+

The proposed structures are compatible with the NMR data if one accepts that the unbound portion of the ligand (namely, the [Phe^4] residue), which is never is a copper chelate ring, is never affected by copper and that the bound residues are broadened when they are in sufficiently rapid exchange for the effect to be transmitted to the excess unbound ligand which is being observed. Selective broadening of tyrosyl nuclei occurs by fleeting interaction of weak-binding sites to vacant axial copper positions. In each case, broadening reflects the presence of very rapidly exchanging minor species.[16] The complications are created by the usual NMR experimental conditions which require large ligand to metal ratios precisely because if no excess ligand is present, the bound molecules are so broadened that no spectrum can be observed. With excess ligand, sec-

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ond-order displacement reactions can occur in addition to formation of "minor species" involving axial coordination. These processes are also responsible for the loss of resolution found in the EPR spectra at copper:enkephalin ratios similar to those used in NMR.

Conclusion

[Leu^5]- and [Me^5]enkephalin binding to copper(II) has been demonstrated in water-simulating physiological serum conditions. As anticipated, complexation is pH dependent and different species are formed under varying conditions. However, at physiological pH, the natural enkephalins form a major species with copper by coordination through the tyrosine amino group, the glycine peptide linkages, and the C-terminal carboxylate functions. No evidence was found for participation of methionine sulfur in copper binding, as both enkephalins showed similar behavior. A well-defined species described by structure II is proposed for the enkephalin–copper complex, despite the difficulties in the determination of binding sites with the experimental conditions used causing interference by minor species. The peptides are strongly held by the metal ions, but the coordination sphere is somewhat flexible. A different kinetically inert complex, structure I, is formed under alkaline conditions; the metal ion is then coordinatively saturated, and the peptides cannot exchange with other potential ligands. These results show that metal ion complexation can eventually lead to conformational information on the mode of action of small biologically active peptides. Correlations between the peptide–metal complex structures and their biological activities could lead to better insight on the conformational requirements at the receptor sites.

Registry No. [Leu^5]enkephalin, 58822-25-6; [Met^5]enkephalin, 58569-55-4; Cu, 7440-50-8.

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Stereochemical Studies on Seven-Coordinate Complexes with Stoichiometry (Bidentate ligand)pentakis(unidentate ligand)metal. Crystal Structures of $[Mo(dppm)(CNCH_3)_5](PF_6)_2$ and $[Mo(dppe)(CNCH_3)_5](PF_6)_2$[1-3]

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The crystal and molecular structures of the two complexes $[Mo(dppm)(CNCH_3)_5](PF_6)_2$ (**1**) and $[Mo(dppe)(CNCH_3)_5](PF_6)_2$ (**2**) are reported [dppm = bis(diphenylphosphino)methane; dppe = 1,2-bis(diphenylphosphino)ethane]. Both compounds contain cations in which a seven-coordinate molybdenum atom is at the center of a distorted pentagonal bipyramid, the pentagonal plane being comprised of three unidentate methyl isocyanide ligands and the bidentate diphosphine ligand. Unidentate methyl isocyanide ligands occupy the remaining two axial sites in both cations. The Mo–C distances range from 2.090 (9) to 2.157 (6) Å in **1** and from 2.096 (7) to 2.150 (5) Å in **2**. The Mo–P distances are 2.519 (1) and 2.538 (1) Å in **1** and 2.518 (1) and 2.557 (2) Å in **2**. The geometries of the cations are compared with those predicted for complexes of stoichiometry (bidentate ligand)pentakis(unidentate ligand)metal by the ligand–ligand repulsion energy calculations of Kepert. Crystal data for **1** are as follows: a = 15.313 (2) Å, b = 17.328 (4) Å, c = 16.891 (3) Å, β = 115.04 (1)°, V = 4060.7 Å^3, Z = 4, monoclinic, Cc, final R = 0.033 for 3380 X-ray diffractometer data with $F_o > 4[\sigma(F_o)]$. Crystal data for **2** are as follows: a = 13.396 (3) Å, b = 14.254 (4) Å, c = 12.740 (2) Å, α = 96.21 (2)°, β = 112.92 (2)°, γ = 99.56 (2)°, V = 2169.1 Å^3, Z = 2, triclinic, $P\bar{1}$, final R = 0.054 for 6007 X-ray diffractometer data with $F_o > 4[\sigma(F_o)]$.

Introduction

Recently we have studied the structures of a variety of seven-coordinate molybdenum(II) and tungsten(II) isocyanide complexes containing unidentate ligands.[5] Criteria were developed for classifying the geometries and analyzing the factors that dictate the choice of stereochemistry for the ML_7^{2+}/ML_6X^+ families, where L = RNC and X = halide or pseudohalide. With the synthesis of the [Mo(diphosphine)(isocyanide)$_5$]$^{2+}$ cations,[6] exploration of related complexes having the stoichiometry [M(BL)(UL)$_5$] (BL = bidentate ligand; UL = unidentate ligand) became possible. In such molecules the constraints of the chelating ligand will influence the structure, and there are highly regarded[7] theoretical models[8,9] for analyzing the resulting stereochemistry.

Here we report the structures of the pentagonal-bipyramidal complexes $[Mo(dppm)(CNCH_3)_5](PF_6)_2$ (**1**)[3] and $[Mo(dppe)(CNCH_3)_5](PF_6)_2$ (**2**)[3] having four- and five-membered chelate rings, respectively. Prior to this study, the only structurally characterized [M(BL)(UL)$_5$] complexes were $[Nb(O_2)F_5]^{3-}$ and $[Ta(O_2)F_5]^{3-}$.[10,11]

Experimental Procedure and Results

Collection and Reduction of X-ray Data. (Bis(diphenylphosphino)methane)pentakis(methyl isocyanide)molybdenum(II) Hexafluorophosphate, $[Mo(dppm)(CNCH_3)_5](PF_6)_2$ (1). The synthesis and crystallization of **1** have been described previously.[6] The orange-red crystal used in the diffraction study had approximate dimensions 0.20 × 0.17 × 0.53 mm and was sealed in a capillary to minimize decomposition. Study on the diffractometer suggested that the crystal belonged to the monoclinic crystal system. Its quality was

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