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QUARTERLY

Effect of tetrazole moiety on coordinating efficiency of deltorphin★

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A study of the effect of the tetrazole moiety, a *cis*-amide bond surrogate, on the Cu(II) coordinating properties of oligopeptides is reported. Insertion of the tetrazole moiety Ψ[CN_4] into the peptide sequence of [D-Ala^2]deltorphin I changes considerably the coordination ability of the peptide. Potentiometric and spectroscopic results show that if the tetrazole moiety is in a suitable position in the peptide chain, i.e. it follows the second residue, a stable CuL species involving 3N coordination is formed in the physiological pH range. The tetrazole Ψ[CN_4] ring provides one of these nitrogens. The data indicate that Cu(II) ions are strongly trapped inside a bent peptide backbone. The peptide conformation changes achieved by Cu(II) coordination may be essential for the binding of tetrazole deltorphins at opiate receptors.

Deltorphins are a family of naturally occurring peptides with high affinity and selectivity for δ opioid binding sites (Melchiorri *et al.*, 1989). They have been targets for many structural modifications and conformational studies. The results revealed that the *cis* Tyr^1-Xaa^2 peptide bond is very likely responsible for the interaction with the receptors and local conformational constrains introduced at the 2- and 3-position residues can also affect the biological activity.

In our studies we modified [D-Ala^2]deltorphin I by inserting 1,5-disubstituted tetrazole ring, which mimics the *cis* amide bond

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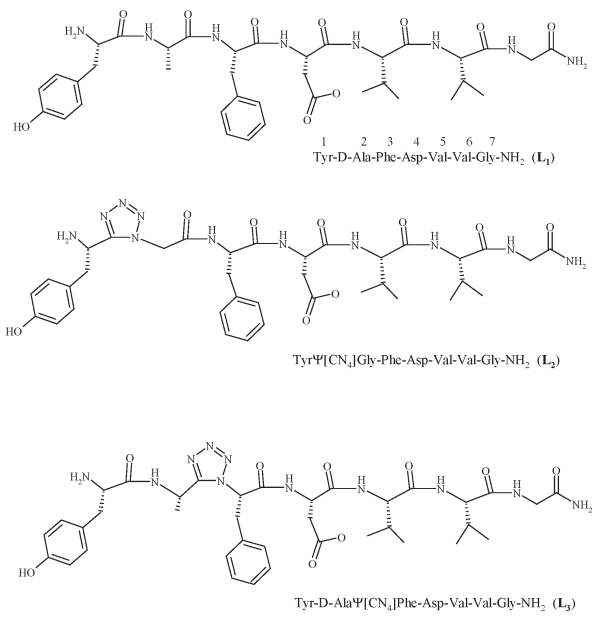
(Zabrocki *et al.*, 1988), between D-Ala^2 and Phe^3, and [Gly^2]deltorphin by inserting the tetrazole ring between Tyr^1 and Gly^2. Receptor binding assays showed a lack of affinity of the tetrazole deltorphin analogues for μ-opioid receptors (Zabrocki & Olczak, unpublished). This may suggest that the tetrazole ring as a mimetic of *cis* amide bond does not invert the μ/δ selectivity in these peptides. The interaction of Cu(II) with deltorphins may have physiological relevance because copper content is especially high in synaptosomal fluids which are rich in opioid peptides (Linder & Goode, 1991). Studies on the Cu(II)-exorphin systems have shown that these exogenous opiate-like peptides are efficient chelating agents (Chruscinska *et al.*, 1997; 1998; Lodyga-Chruscinska *et al.*, 1999). Moreover, insertion of the tetrazole ring can effectively stabilize the metallopeptide structure (Lodyga-Chruscinska *et al.*, 1999; 2000; Chruscinska *et al.*, 2001). These results led us to study metal complexes of several deltorphin derivatives (Scheme 1) in order to evaluate the factors governing their chelating ability. The study herein reported was performed on Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2 (**L_1**), TyrΨ[CN_4]Gly-Phe-Asp-Val-Val-Gly-NH_2 (**L_2**) and Tyr-D-AlaΨ[CN_4]Phe-Asp-Val-Val-Gly-NH_2 (**L_3**) – copper(II) systems by integrating several spectroscopic techniques (UV-Vis, CD and EPR), combined with potentiometric measurements and computer modelling optimisation.



Scheme 1. [D-Ala^2]deltorphin I and its two tetrazole analogues.

MATERIALS AND METHODS

Reagents. The tetrazole deltorphin derivatives were synthesized according to a previously reported procedure (Zabrocki *et al.*, 1988). Their purity was verified by HPLC (high performance liquid chromatography), mass spectrometry and potentiometry to be >99%. [D-Ala^2]deltorphin I was purchased from Bachem and used without purification, $Cu(NO_3)_2$, KNO_3, HNO_3 and NaOH were Merck products and used without any further purification.

Potentiometric studies. Protonation and coordination equilibria were investigated by potentiometric titration in aqueous solution, over the pH 3–11 range, at a constant ionic strength using 0.1 M KNO_3 and at constant temperature (298 K) under argon atmosphere with a total volume of 1.5–2 cm^3. A 0.05 M solution of $Cu(NO_3)_2$ was used as the stock for the Cu(II) ion. An automatic titration set including autoburette meter (Molspin Ltd., Newcastle-upon-Tyne, U.K.), a semi-micro-combined electrode (Russell CMAWL/S7) and an IBM-compatible PC were used to collect data. Alkali, about 0.1 M NaOH, free of CO_2 was added from a 0.250 cm^3 micrometer syringe, which was calibrated by both weight titration and titration of standard materials. The electrode was calibrated for hydrogen ion activity. The relationship between activity and concentration was calculated daily by titration with HNO_3 (Irving *et al.*, 1967). Calculations were made with the aid of SUPERQUAD computer program (Gans *et al.*, 1985). This allows the refinement of total ligand concentrations. Therefore it was able to confirm

the purity of the peptide, in particular the absence of acetate, a frequent impurity in peptide samples, or other coordinating ions. In all cases triplicate titrations (about 500 experimental points in one set of measurements) were carried out at Cu/L ratio 1:1. The ligand concentration was 1×10^{-3} M. As usual, the stabilities of the metal complexes are reported as the logarithms of the overall formation constants $\beta_{pqr} = [M_pH_qL_r]/ [M]^p[H]^q[L]^r$, where M stands for the metal ion, H is proton and L the deprotonated form of the ligand (Table 1). The standard deviations quoted were computed by SUPERQUAD and refer to random errors only. They are, however, a good indication of the importance of a particular species in the equilibrium.

Spectroscopic studies. Electronic absorption spectra (UV-Vis) were recorded with a Perkin-Elmer Lambda 11 spectrophotometer. Circular dichroism (CD) spectra were obtained with a Jobin-Yvon CD-6 dichrograph over the range 200–750 nm, using 1 and 0.05 cm cuvettes. The spectra are expressed as $\Delta\varepsilon = \varepsilon_l - \varepsilon_r$, where ε_l and ε_r are the molar absorption coefficients for left and right circularly polarized light, respectively. Electron paramagnetic resonance (EPR) measurements were carried out with a Varian E-9 instrument at the X-band frequency (9.1 GHz) at 120 K; about 10% of ethanediol was added to the samples in order to obtain good glasses. Measurements were performed at the maximum concentration of each species found in titrations. The EPR parameters were read from the spectra (estimated uncertainties for A and g values are 1×10^{-4} cm^{-1} and 0.002, respectively, in the spectra of single species).

Table 1. Stability constants (log β_{pqr}) for $Cu_pH_qL_r$ systems at 298 K and I = 0.1 M (KNO_3)

Ligand Species *pqr*	log β	$pK_{COOH(Asp)}$	$pK_{NH_2(Term)}$	$pK_{OH(Tyr)}$
Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2 (L_1)		4.08	7.63	9.97
HL	9.97(1)			
H_2L	17.60(1)			
H_3L	21.68(1)			
CuHL	15.48(1)			
CuL	9.23(1)			
$CuH_{-1}L$	1.96(1)			
$CuH_{-2}L$	–5.81(2)			
$CuH_{-3}L$	–16.02(1)			
TyrΨ[CN_4]Gly-Phe-Asp-Val-Val-Gly-NH_2 (L_2)		3.80	5.47	9.79
HL	9.79(29			
H_2L	15.26(3)			
H_3L	19.06(3)			
CuHL	12.97(2)			
CuL	6.79(2)			
$CuH_{-1}L$	0.82(1)			
$CuH_{-2}L$	–9.01(2)			
Tyr-D-AlaΨ[CN_4]Phe-Asp-Val-Val-Gly-NH_2 (L_3)		3.89	6.92	9.92
HL	9.92(1)			
H_2L	16.84(1)			
H_3L	20.73(1)			
CuHL	14.25(3)			
CuL	10.02(1)			
$Cu_2H_{-2}L_2$	5.77(2)			
$CuH_{-2}L$	–9.03(1)			

Computational studies. All calculations were carried out using the MOPAC 2002 program (MOPAC, 2002). The geometries of all ligands were fully optimized using the semi-empirical molecular orbital method AM1 (Dewar *et al.*, 1985). The copper ion complexes were fully geometry optimized using unrestricted Hartree-Fock approximation, assuming the open shell electronic state of a molecule as a doublet and using the AM1d method to treat the copper ion (Voityuk Rosch, 2000), the solvent (water) environment was simulated by employing the COSMO method (Klamt & Schurman, 1993). Our previous study shows that the AM1d method reasonably well describes the geometries and energetics of peptide copper ion complexes (Brasun *et al.*, 2000; 2001; Chruscinska *et al.*, 2003). A systematic search of the conformational space of free ligands, copper (II) complexes with full length ligands and copper (II) complexes with short ligands (the C-terminal tripeptide (Val-Val-Gly-NH_2) was replaced by the NH_2 moiety) was performed, about 300 different conformations for every system were generated and fully geometry-optimized and the nature of the stationary point was determined by normal mode analysis. A typical geometry optimization plus frequency calculation run took from 1 to 3 h of computation time on PIII XEON 700MHz CPU.

RESULTS AND DISCUSSION

Protonation equilibria of free peptide

Protonation constants for the peptides studied (Scheme 1) are presented in Table 1.

The ligands have three groups, N-terminal amine, Asp carboxylate and Tyr phenolate, which are capable of reversible proton binding. The lowest and the highest pK_a values can be assigned to p$K_{COOH(Asp)}$ and p$K_{OH(Tyr)}$ of the Asp and Tyr side chains, respectively, and they are very close to those measured for other oligopeptides (Decock-Le Reverend *et al.*, 1986; 1986; Galey *et al.*, 1991; Lodyga-Chruscinska *et al.*, 1999; Kowalik-Jankowska *et al.*,1999, 2000). The p$K_{NH_3^+}$ values for $\mathbf{L_1}$ and $\mathbf{L_3}$, are typical for the NH_2-terminal group (Sovago, 1990). The $\mathbf{L_2}$ case is different: insertion of the tetrazole moiety between Tyr^1 and Gly^2 has a significant effect on the acidity of the amino group. Here, p$K_{NH_3^+}$ is lower by 2.16 units compared to the parent deltorphin and by about 1.45 units compared to the other tetrazole deltorphin analogue (Table 1). This is an effect of the electron-withdrawing properties of the 1,5-disubstituted tetrazole ring. This effect is much smaller in $\mathbf{L_3}$ because the terminal amino nitrogen is further removed from the Ψ[CN_4] moiety. Hence the protonation constant of the N-terminal tyrosine residue is closer to that found for the other tetrazole peptide analogues (Lodyga-Chruscinska *et al.*, 1999; 2000).

Complex formation

The species distribution diagrams indicate the formation of five Cu(II) complex species in the case of $\mathbf{L_1}$, and four species in the cases of $\mathbf{L_2}$, $\mathbf{L_3}$ (Fig. 1).

The stability constants (log β) and spectroscopic parameters of the complexes formed are given in Tables 1 and 2, respectively.

The peptide Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2 ($\mathbf{L_1}$)

The coordination binding starts at the N-terminal amino group site and follows by sequential deprotonation of neighboring peptide nitrogens. The first complex to form, starting at low pH, is CuHL (Fig. 1) which may be regarded as comparable to the complex of simple oligopeptides (Bal *et al.*, 1993) with an additional protonated phenolic group of the Tyr residue. This species can be supported by spectroscopic data with the typical d-d transition at 721 nm (Table 2) characteris-

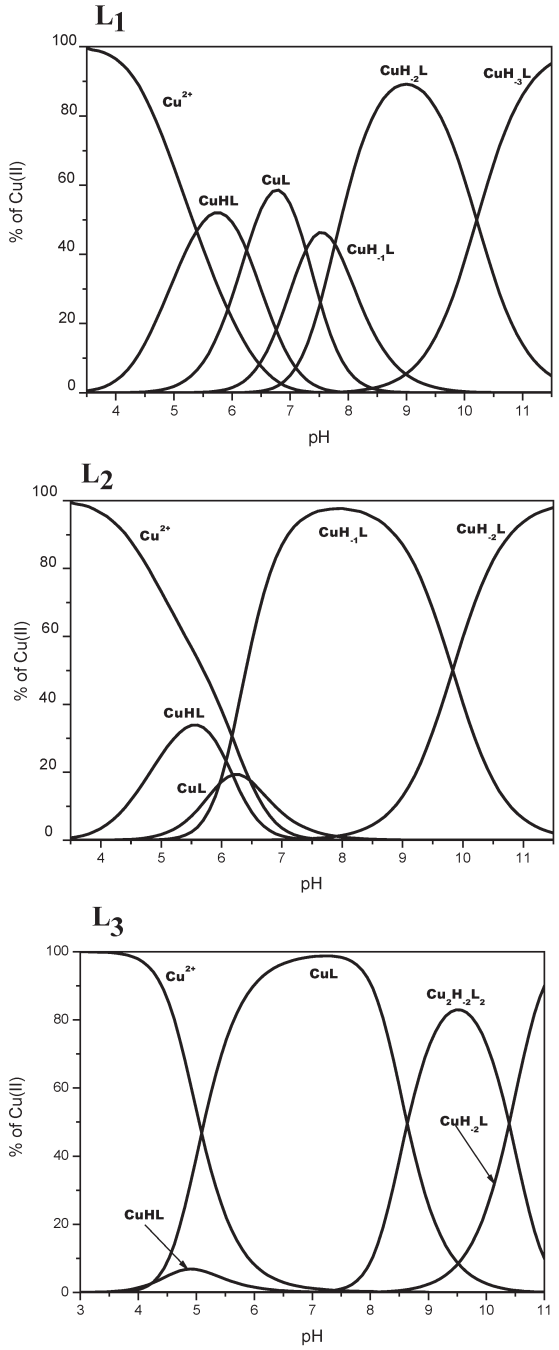


Figure 1. Species distribution curves for Cu(II)-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2 (L_1), TyrΨ[CN_4]Gly-Phe-Asp-Val-Val-Gly-NH_2 (L_2), Tyr-D-AlaΨ[CN_4]Phe-Asp-Val-Val-Gly-NH_2 (L_3) complexes at 298 K and I = 0.1 M KNO_3.

Metal to ligand ratio 1:1; metal concentration 1 mM.

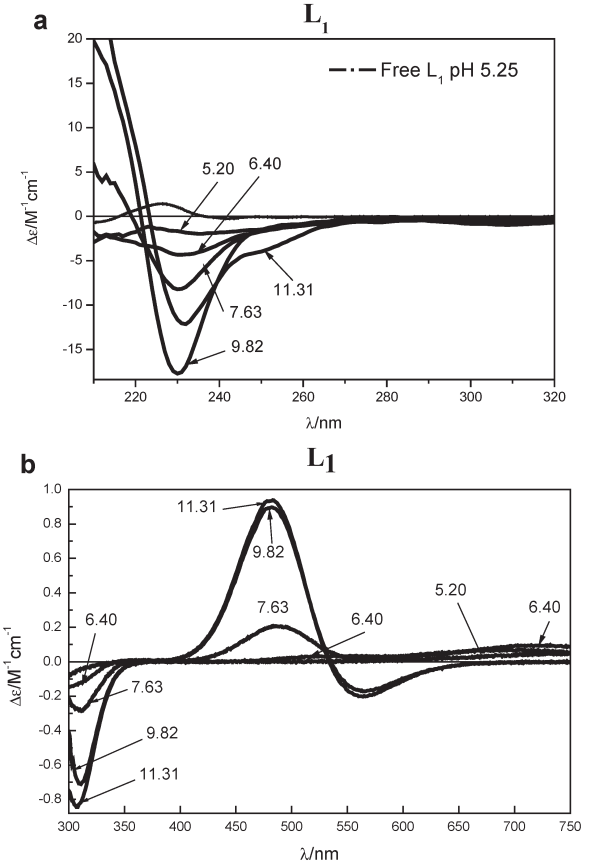


Figure 2. UV (a) and visible (b) CD spectra of Cu(II)-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2 (L_1) system as a function of pH.

Ligand to metal ratio 1:1; ligand concentration 1 mM.

tic for 1N coordination (Kozłowski & Micera, 1995). With increasing pH this complex loses the amide proton to give a species of CuL stoichiometry. The d-d transition energy 653 nm (Table 2) and the presence in the CD spectra of an $N^- \rightarrow Cu^{2+}$ charge transfer transition at 300 nm (Fig. 2) are consistent with 2N coordination {NH_2, N^-, CO} of the peptide to copper(II) ions (Pettit *et al.*, 1990). At pH 6.5–8.5, the $CuH_{-1}L$ complex with {NH_2, $2N^-$, CO}coordination is formed. This binding mode is easily seen in the absorption and CD spectra (Table 2, Fig. 2). The d-d transition at 540 nm and the parameters of CD spectra correspond to the 3N binding mode (Pettit *et al.*, 1990). Above pH 7 this complex loses the next amide proton to give a $CuH_{-2}L$ species with four nitrogen donors arranged in a square planar geometry around the central copper(II) ion. The shift of absorption maximum to 507 nm in the electronic absorption spectra and the CD data support the involvement of a fourth nitrogen donor atom (Table 2, Fig. 2). The pH increasing above 9 results

in the formation of $CuH_{-3}L$ species. This complex has a similar binding mode to that of $CuH_{-2}L$, except for the deprotonated phenolate. Accordingly, the CD and absorption parameters of $CuH_{-3}L$ are similar to those obtained for the $CuH_{-2}L$ complex (Table 2, Fig. 2). The phenolic O^- of the Tyr residue does not coordinate to the metal ion which is supported by complete absence of the characteristic charge transfer band in the CD spectrum at 400 nm (Fig. 2) (Livera *et al.*, 1988). The protonation constant of $[CuH_{-3}L]$ (10.21, log value) is comparable to that of the Tyr residue in the free ligand (9.97) (Table 1).

Table 2. Spectroscopic parameters for deltorphin I and its two tetrazole derivatives

Cu:L = 1:1; c_{Cu} = 1 mM; I = 0.1 M (KNO_3) ; T = 298 K.

Ligand	pH	EPR		UV-Vis	
Species {donor set}	range	$g_{\|}$	$A_{\|}$ /$10^{-4}cm^{-1}$	λ /nm	ε /$M^{-1}cm^{-1}$
Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2					
CuHL {NH_2,CO}	4.5–6.5			721	38
CuL {NH_2,N^-,CO}	6–7.5			653	74
$CuH_{-1}L$ {NH_2,N^-,N^-,CO}	7–8.5			540	107
$CuH_{-2}L$ {NH_2,N^-,N^-, N^-}	8–10			507	167
$CuH_{-3}L$ {NH_2,N^-,N^-, N^-}	9.5–12			507	173
TyrΨ[CN_4]Gly-Phe-Asp-Val-Val-Gly-NH_2					
CuHL {NH_2, N_{tetr}}	4.5–6			670	49
CuL {NH_2, N^-}	5.5–7			596	51
$CuH_{-1}L$ {NH_2,N^-,N^-,COO^-}	7–9			574	90
$CuH_{-2}L$ {NH_2,N^-,N^-,COO^-}	10–12			553	97
Tyr-D-AlaΨ[CN_4]Phe-Asp-Val-Val-Gly-NH_2					
CuL {NH_2,N^-,N_{tetr}}	4.5–9.0	2.233	185	614	75
$Cu_2H_{-2}L_2$ {NH_2,N^-,N_{tetr}, O^-_{Tyr}}	8.5–10.5	–	–	611	94
				393	390
$CuH_{-2}L$ {NH_2,N^-,N^-}	10–12	2.229	179	565	101

The peptide TyrΨ[CN_4]Gly-Phe-Asp-Val-Val-Gly-NH_2 (**L_2**)

This peptide exhibits a coordination behavior different from that of other tetrazole analogues (Lodyga-Chruscinska *et al.*, 1999; 2000; Chruscinska *et al.*, 2001). Over the low pH region CuHL is a minor species (Fig. 1), nevertheless it can be detected spectroscopically. The spectroscopic parameters of this complex are intermediate between those of the 1N and 2N complexes, suggesting the coordination of the terminal amine nitrogen and, most likely, tetrazole nitrogen (Table 2). The CD spectrum of the fully protonated form of **L_2** exhibits two negative bands over the 200–320 nm range, one above 200 nm and the another above 230 nm originating from the peptide chromophores (Woody, 1985) (Fig. 3a).

As a result of the CuHL formation, the CD spectrum of the ligand shows a significant increase of the Cotton effect at about 200 nm and the appearance of optical activity at 270 nm (Fig. 3a). The donor set is very likely because the d-d transition energy of the CD spectrum of CuHL complex is close to that ex-

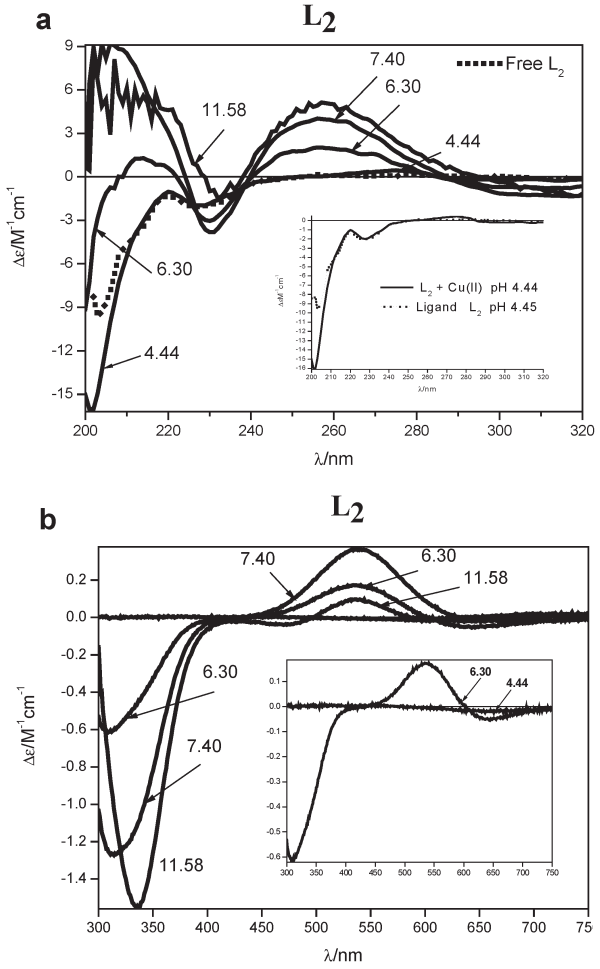


Figure 3. UV (a) and visible (b) CD spectra of Cu(II)-TyrΨ[CN_4]Gly-Phe-Asp-Val-Val-Gly-NH_2 (L_2) as a function of pH.

Ligand to metal ratio 1:1; ligand concentration 1 mM.

pected for 2N coordination, and in addition the amide nitrogen coordination is not supported by the presence of a CT band around 310 nm (Kozłowski & Micera, 1995). The stability constant of this complex, corrected for the ligand basicity which can be assumed as a quantification of complex stabilization (Kozlowski *et al.*, 1999), is comparable to that of CuHL species of **L_1** (Table 3).

It means that the involvement of the tetrazole nitrogen in copper binding has a minor effect in this complex stabilization. The species CuL is formed in a more alkaline solution. It can be supported by CD spectra which display significant changes both in the UV and visible regions with comparison to those of the CuHL complex. A noticeable decrease in the first negative Cotton effect at around 200 nm, some increase in the second one at 228 nm and the appearance of a new positive Cotton effect in 260 nm region can be seen (Fig. 3). The latter effect is characteristic for charge transfer transition of β-carboxylate oxygen of the Asp residue (Kozłowski & Micera, 1995; Daniele *et al.*, 1996). The band is distinctly broad indicating overlap transitions. On the other hand, the d-d transition band and the $N^- \rightarrow Cu(II)$ charge transfer at 310 nm strongly support the {NH_2, N^-} coordination (Kozłowski *et al.*, 1995). The N^- coordination is substantiated by the pK_a for the amide deprotonation (pK_a = 6.18) which is close to that found for unmodified deltorphin (pK_a = 6.25). The species dominating in the pH range 6.5–9.5 is the $CuH_{-1}L$. Its formation can be attributed to the deprotonation of the second amide nitrogen. The d-d transition at 574 nm which is close to the value predicted by the equation: $\lambda_{max} = 10^3/[1.18 + 0.166(NH_2) + 2 \times 0.200(N=)] \approx 573$ nm supports the 3N coordination (Sigel & Martin, 1982). A band at 260 nm in the CD spectrum (Fig. 3a) suggests an involvement of Asp β-carboxylate in Cu(II) binding (Decock-Le Reverend *et al.*, 1986; 1986; Daniele *et al.*, 1996). The equatorial coordination of the $CuH_{-1}L$ complex involves the N-terminal amino group, two amide nitrogens of the Phe and Asp residues and the β-carboxylate oxygen of the Asp residue yielding two chelated rings, eight and five-membered, respectively. The stability constant of this complex is 1.24 log units greater than that of the same species of the **L_1** ligand (Table 3). This results in the dominance of $CuH_{-1}L$ at pH 7–10 region. Above pH 10 $CuH_{-2}L$ is formed. The log value for the protonation constant of this complex is 9.83 which is very close to that of the Tyr side chain (9.79). The spectroscopic parameters still support the {NH_2, $2N^-$, COO^-} coordination mode (Table 2). The blue shift of the absorption band to 553 nm and the changes in the intensities of the Cotton effects in CD

Table 3. Protonation corrected stability constants ($\log^{*}K = \log \beta - \log \beta H_2L$).

Ligand	Species	$\log^{*}K$
Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2	CuHL {NH_2, CO} 1N	–2.12
	CuL {NH_2, N^-, CO} 2N	–8.37
	$CuH_{-1}L$ {NH_2, N^-, N^-, CO} 3N	–15.64
	$CuH_{-2}L$ {NH_2, N^-, N^-, N^-} 4N	–23.41
TyrΨ[CN_4]Gly-Phe-Asp-Val-Val-Gly-NH_2	CuHL {NH_2, N_{tetr}} 2N	–2.29
	CuL {NH_2, N^-,CO} 2N	–8.47
	$CuH_{-1}L$ {NH_2, N^-, N^-, COO^-} 3N	–14.44
	$CuH_{-2}L$ {NH_2, N^-, N^-, COO^-} 3N	–24.27
Tyr-D-AlaΨ[CN_4]Phe-Asp-Val-Val-Gly-NH_2	CuHL {NH_2, CO} 1N	–2.59
	CuL {NH_2, N^-, N_{tetr}} 3N	–6.82
	$CuH_{-2}L$ {NH_2, N^-, N^-} 3N	–25.85

spectra (Fig. 3) may reflect some constraints within the peptide backbone due to intra-ligand interactions following the deprotonation of the Tyr phenolic group.

The peptide Tyr-D-AlaΨ[CN_4]Phe-Asp-Val-Val-Gly-NH_2 (L_3)

According to the potentiometric data the **L_3** analogue gives four complex species: CuHL, CuL, $Cu_2H_{-2}L_2$ and $CuH_{-2}L$. (Fig. 1, Table 1). The minor species CuHL with {NH_2, CO} coordination, usually found in metal-peptide systems (Sovago, 1990; Kozłowski & Micera, 1995; Lodyga- Chruscinska *et al.*, 1999, 2000), cannot be evidenced by spectroscopic data because of its very low concentration. The CuL species dominates around physiological pH. The d-d transition energy at 614 nm, the EPR parameters $g_{\|}$ = 2.232 and $A_{\|}$ = 185 × $10^{-4}cm^{-1}$ (Table 2) and the $NH_2 \rightarrow$Cu(II) and $N^- \rightarrow$Cu(II) charge transfer transitions at 273 and 304 nm, respectively, in the CD spectra (Fig. 4) are consistent with the {NH_2, N^-, N_{tetr}} coordination (Lodyga-Chruscinska *et al.*, 1999, 2000; Chruscinska *et al.*, 2001).

Above pH 8 the $O^-_{Tyr} \rightarrow$Cu(II) charge transfer transition shows up in the CD and absorption spectra, whereas the EPR signal intensity is reduced, which suggests a magnetic interaction between copper(II) ions in the complex (Fig. 5, Table 2). The potentiometric data are in good agreement with the spectroscopic results and indicate a dimeric $Cu_2H_{-2}L_2$ species with {NH_2, N^-, N_{tetr}, O^-_{Tyr}} coordination. This binding mode is supported by the electronic absorption and CD spectra (Herfford & Pettit, 1981). The deprotonated phenolate oxygens act as bridging ligands as it occurs in some tetrazole peptide analogues (Lodyga-Chruscinska *et al.*, 1999; 2000). Dimer formation observed only in this system arises from a specific peptide conformation. Conformational analysis of deltorphin has in-

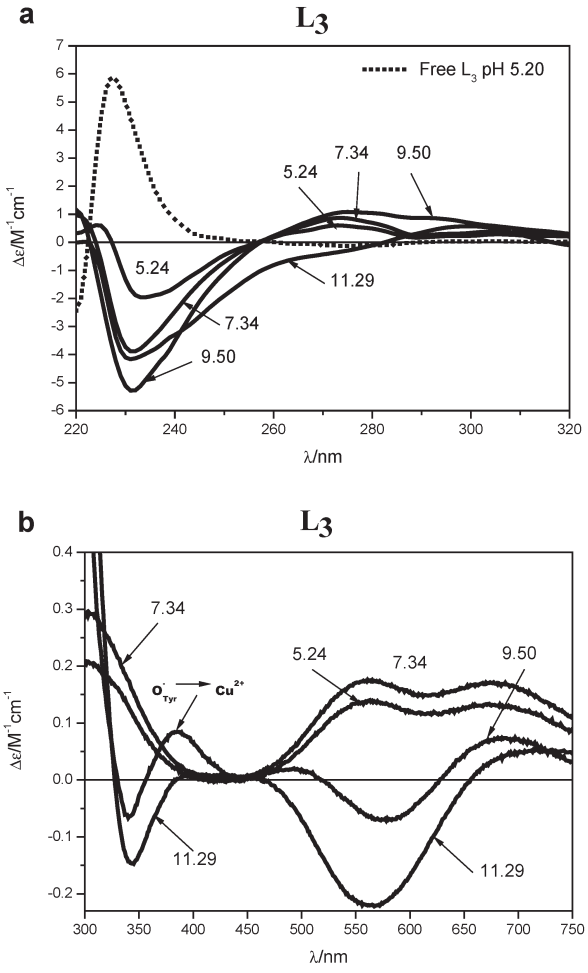


Figure 4. UV (a) and visible (b) CD spectra of Cu(II)-Tyr-D-AlaΨ[CN_4]Phe-Asp-Val-Val-Gly-NH_2 (L_3) system as a function of pH.

Ligand to metal ratio 1:1; ligand concentration 1 mM.

dicated that [D-Ala^2]deltorphin I can adopt a folded β-turn-type structure (Bryant *et al.*, 1994). The insertion of tetrazole moiety after the D-Ala residue stabilizes such a folded conformation (Zabrocki *et al.*, 1998; Zabrocki & Olczak unpublished) that is more favorable for bridging two Tyr residues to form a dimeric species. Above pH 10 for the Cu(II)-$\mathbf{L_3}$ system, the band indicating the phenolate oxygen binding is no longer observed and the $CuH_{-2}L$ complex with the Tyr side chain deprotonated is formed. The distinct intensity increase of the $N^- \rightarrow Cu(II)$ charge transfer band (above 310 nm) seen in the CD spectrum (Fig. 4) suggests that in this monomeric complex additional amide nitrogens are involved in the metal coordination. The same binding pattern was observed for tetrazole enkephalin analogues, in which the tetrazole ring inserted into the peptide sequence acts as a break-point (Lodyga-Chruscinska *et al.*, 1999). The N_{tetr} donor is weakly basic and thus sensitive to hydrolysis at higher pH. Therefore the Cu(II)-N_{tetr} bond can be replaced by the amide nitrogen of the Asp residue resulting in the $\{NH_2, 2N^-\}$ coordination mode. Electronic absorption, EPR and CD spectra (Table 2, Fig. 4) support this 3N coordination mode. The red shift of electronic absorption spectra and the decreased parameters of EPR seem to support the involvement of an OH^- group from deprotonated water molecule in Cu(II) coordination (Chruscinska *et al.*, 2001).

Theoretical calculations

Analysis of free ligands

Conformational analysis of free ligands shows that all of them display the conformational flexibility typical for the short peptides and this feature is not affected by the introduction of the tetrazole moiety into the peptide backbone. The unusual value of pK for the amino group in the $\mathbf{L_2}$ ligand (see Table 2) cannot be associated with the formation of an intramolecular hydrogen bond between the protons from the amino group and the tetrazole nitrogen atoms. The distance between the protons from the N-terminal amino group and tetrazole nitrogen is about 2.7 Å, which corresponds to a very week interaction. Thus this very unusual pK value should rather be associated with the presence of the tetrazole moiety in a close proximity of the protonated amino group and an electron withdrawing effect. The hydrogen atoms in the amino group of the $\mathbf{L_2}$ ligand have a net charge of +0.2771e (Mullican population) but in the case of other ligands ($\mathbf{L_1}$, $\mathbf{L_3}$) the net charge is only about +0.2617 ± 0.002e. In the case of all ligands ($\mathbf{L_1}$, $\mathbf{L_2}$ and $\mathbf{L_3}$) the net

charge of the nitrogen atom remains almost unchanged and it is equal to −0.0585 ± 0.002e. This result shows that the N-H bonds are more polarized in the case of the **L_2** ligand and this increases the tendency of the amino proton to dissociate at lower pH.

Analysis of copper ion complexes

Conformational analysis of copper ion complexes with all ligands shows that the C-terminal tripeptide Val-Val-Gly-NH_2 is very flexible and has a very week influence on the coordination sphere of the central ion. In Fig. 5 the lowest energy conformation of copper ion complexes with short ligands (without the C-terminal tripeptide) are shown.

In all the complexes the coordination sphere is square planar with three or four ligand moieties interacting with the central ion. The observed Cu-N distance varied from 1.88 to 2.1 Å and Cu-O distance varied from 1.85 to 2.2 Å which is in very good agreement with the distances observed in X-ray structures of similar complexes found in the CSD database (Allen *et al.*, 1983). Figure 5a shows the structure of the $CuH_{-2}L$ complex formed by the **L_1** ligand. We can observe an almost ideally planar (the deviation from planarity is less than 0.3^o) structure of the coordination sphere around the central ion formed by four nitrogen atoms, one from N-terminal amino group and deprotonated amides. The whole structure is stabilized by a hydrophobic cluster formed by the side chains of phenylalanine from position 3 and tyrosine from position 1 and by electrostatic interaction between the N-terminal amino group and the carboxylic group from the aspartic acid at position 4.

Figure 5b shows the structure of the complex $CuH_{-1}L$ formed by the **L_2** ligand. The coordination sphere around the central ion is square planar and it is filled by three nitrogen atoms and by oxygen from the carboxylic group of aspartic acid. We can observe that the introduction of the tetrazole moiety into the peptide backbone generated a very small

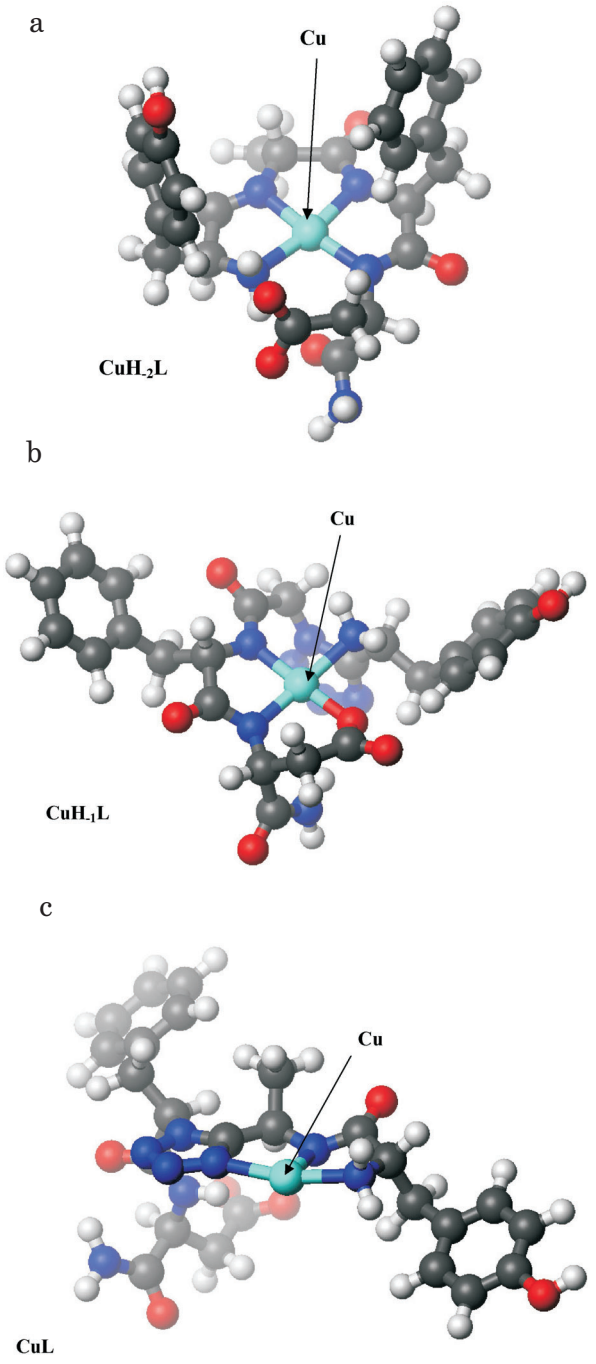


Figure 5. The lowest energy conformations of complexes of copper(II) and [D-Ala^2]deltorphin I and its analogues in our theoretical studies for:

(a) Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2 (**L_1**), (b) TyrΨ-[CN_4]Gly-Phe-Asp-Val-Val-Gly-NH_2 (**L_2**), and (c) Tyr-D-AlaΨ[CN_4]Phe-Asp-Val-Val-Gly-NH_2 (**L_3**).

distortion (0.2^o) in the cooplanarity of the coordination sphere of the central ion. We present the lowest energy conformation in which the hydrophobic interactions between the

side chains of Tyr^1 and Phe^3 observed in the previously described complex, are now destroyed by the introduction of the tetrazole ring which leads to a change of the peptide backbone geometry and pushes both side chains far apart. The introduction of the tetrazole ring between the positions 1 and 2 creates an unusual turn-like conformation of the peptide backbone. This conformation brings to a very close proximity three groups of atoms participating in the coordination of the central metal ion (N-terminal amino group, tetrazole ring and amide nitrogen from the peptide group between residues 2 and 3). This observation can explain why in the potentiometric experiment we observed a low concentration of the CuHL species (see Fig. 1). The close spatial proximity of the tetrazole ring and the peptide group between the residues 2 and 3 allow a very fast transition between the CuHL and CuL complexes without significant changes in the ligand conformation. Similarly, as it is shown in Fig. 1, the CuL complex is poorly represented and we should expect a very fast transition between CuL and $CuH_{-1}L$ as well.

Figure 5c shows the structure of the CuL complex formed by the **L_3** ligand (short form). The coordination sphere around the central ion is formed by three nitrogen atoms (N-terminal amino group, deprotonated amide nitrogen and tetrazole nitrogen) and in water solution the fourth coordination site is occupied by a water molecule or hydroxyl ion. In our calculations we do not use the explicit solvent and this is the reason why in our model this coordination site remains empty. In the case of the previously described ligand (**L_2**), the fourth coordination site is filled by carboxyl group. In the case of **L_3** such an interaction is impossible because the tetrazole ring located between residues 2–3 pushes the C-terminal tail of the peptide far away from the central ion. The presence of an unoccupied coordination site can explain the suggestion coming from potentiometric experiments about formation of dimeric complexes by **L_3**. The structure presented in Fig. 5c can be interpreted easily as monomer in a dimer complex. We can see in Fig. 5c that the side chain of tyrosine from position 1 is located above the coordination plane. The phenolic oxygen cannot coordinate to the central ion of the same molecule but can easily coordinate ion from another molecule and simultaneously the phenolic oxygen from the second molecule can coordinate to the central ion of the first one and form the dimer, as deduced from potentiometric measurements (see Fig. 1). In the case of **L_3** the CuL complex dominates in the pH range 5–9 and $CuH_{-1}L$ is not detected by spectroscopy. As shown in Fig. 5c, the CuL complex of the **L_3** cannot be easily transformed into the $CuH_{-1}L$ complex. The presence of the methyl from the D-alanine residue in position 2 prevents free rotation around the bond between the α-carbon from Ala^2 and N1 nitrogen from tetrazole ring. Such a rotation is necessary to remove the tetrazole ring from the coordination sphere of the central ion and to bring about the amide nitrogen from residue 4 in a close proximity of the copper ion and subsequently the transformation of CuL into $CuH_{-1}L$. A sterical repulsion between the methyl group from residue 2 and tetrazole ring creates a kinetic trap which stabilizes the CuL complex formed by the **L_3** analogue. The plot demonstrating competition between **L_3** and **L_1** ligands towards Cu(II) ions shows that in the pH range 6–8 the parent peptide coordinates only about 20% of total copper (Fig. 6).

This indicates that at the physiological pH range the tetrazole analogue is more effective in forming the 3N species than [D-Ala^2]deltorphin I itself.

The potentiometric, spectroscopic and theoretical studies reported here show that 1,5-disubstituted tetrazole ring strongly influences the coordinating ability of the peptides studied. The tetrazole nitrogen can be involved in metal binding as a donor atom. It is also interesting to note that tetrazole moiety can stabilize a folded peptide conformation

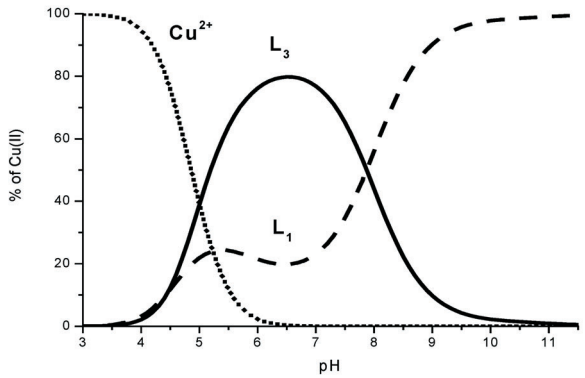


Figure 6. Competition diagram for (L_3) tetrazole analogue (solid line) and [D-Ala^2]deltorphin I (L_1) (dashed line) in binding of Cu(II) ions as a function of pH for 1:1:1 (ligand/metal/ligand) molar ratio and metal concentration 1 mM

that is more favorable for bridging two Tyr residues to form a dimeric species.

To summarize, one can conclude that conformation changes caused by Cu(II) coordination may be essential for the binding of tetrazole deltorphins at opiate receptors.

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