Specific Binding of the Tyrosine Residue in Copper(II) Complexes of Tyr-Pro-Gly-Tyr and Tyr-Gly-Pro-Tyr

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The syntheses of the tetrapeptides Tyr-Pro-Gly-Tyr and Tyr-Gly-Pro-Tyr (H_3L) are reported, together with the results of a potentiometric and spectrophotometric study of their H^+ and Cu^{2+} complexes. Proline acts as a break-point to metal-ion co-ordination when inserted into a peptide chain and in Tyr(1)-Pro(2)-Gly(3)-Tyr(4) [Pro(2)] the Pro residue enforces a bent conformation and the formation of an unusually stable [CuHL] complex with co-ordination through the terminal amine-N of Tyr(1), the neighbouring peptide =CO, and the O_{Tyr}^- of Tyr(4). This necessitates a 17-membered chelate ring. With Tyr-Gly-Pro-Tyr [Pro(3)] there is also O_{Tyr}^- –C bonding but this is a result of dimer formation in $[(CuL)_2]^{2-}$

The proline residue (Pro) acts as a break-point to Cu^{2+} ion co-ordination when inserted into an oligopeptide chain;[1,2] it also encourages the formation of β-turns in the peptide chain and hence a horse-shoe shaped conformation.[3] As a result complexes are formed at intermediate pH which contain large (11-membered) chelate rings which bridge the two ends of a chain, locking the peptide into a bent conformation typical of many biologically-active oligopeptides.[1]

Many neuropeptides contain the tyrosine residue (Tyr), *e.g.* all enkephalins,[4] and the brain has an unexpectedly high concentration of copper to which no role can be given at the moment. The phenolic proton of Tyr does not normally ionize until above pH 10. In tyrosine itself, O_{Tyr}^- (the phenolate oxygen) cannot co-ordinate to Cu^{2+} ions already bonded to the $-NH_2$ group for steric reasons but interaction is possible in complexes of dipeptides such as Tyr-Gly (Gly = glycine residue) through dimer formation.[5] This interaction can be detected most simply from the associated charge-transfer band in the absorption spectrum at *ca.* 400 nm.[6] A study of tetrapeptides based on Tyr-Pro-Gly-Gly with Tyr in positions 1, 3, and 4 has shown that dimerisation can occur but did not pursue the influence of the position of the Pro residue on the species formed.[6]

We wish to report the synthesis of the tetrapeptides L-Tyr(1)-L-Pro(2)-Gly(3)-L-Tyr(4) and L-Tyr(1)-Gly(2)-L-Pro(3)-L-Tyr(4) to allow a study of the effect of moving Pro from position 2 to position 3, and possible co-operative effects resulting from Tyr in positions 1 and 4. The complexes with Cu^{2+} have been studied potentiometrically and spectrophotometrically, using absorption, circular dichroism (c.d.), and e.s.r. spectroscopy. From the potentiometric studies it was possible to identify the species present in the equilibria and calculate reliable species distribution curves. Comparison of calculated formation constants with those for similar complexes of known structure gives information on the structures of the species. The spectroscopic studies, particularly c.d. spectroscopy, gave more precise information on the co-ordination centres involved[7-9] and e.s.r. spectroscopy was used to study dimer formation.

Experimental

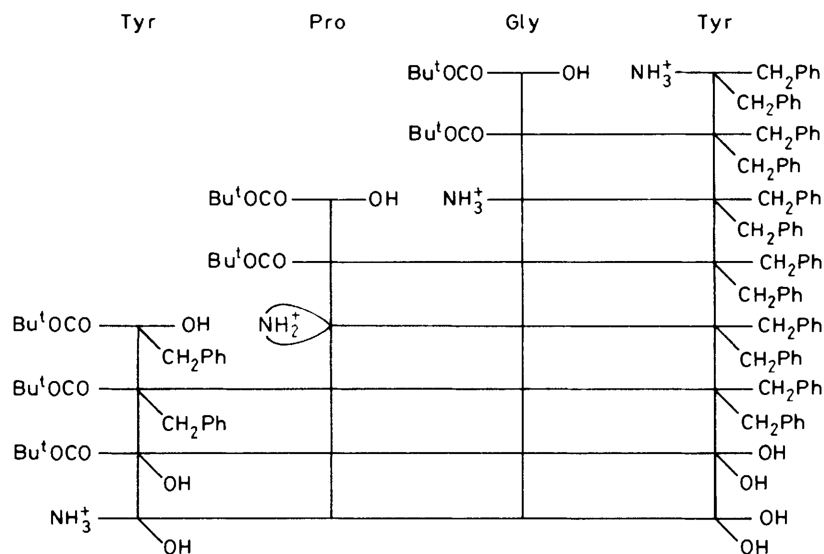
Organic Syntheses.—The tetrapeptides were synthesized by standard liquid-phase methods. The starting materials were Bu^tOCO-Gly, Bu^tOCO-L-Pro, Bu^tOCO-Tyr(CH_2Ph), and Tyr(CH_2Ph)–CH_2Ph·HCl.

The synthesis of Tyr-Pro-Gly-Tyr is outlined in the Scheme; for the synthesis of Tyr-Gly-Pro-Tyr, Gly and Pro are interchanged.

C-Protected derivatives (in chloroform solution) were neutralized with triethylamine before coupling. The coupling reagents were dicyclohexylcarbodi-imide (dcci, Merck) and 1-hydroxybenzotriazole (Aldrich). Benzyl groups were removed by hydrogenolysis using 10% Pd on charcoal as catalyst. The Bu^tOCO groups were cleaved using HCl (4 mol dm^{-3}) in dioxane. Tetrapeptides were purified by gel filtration (Sephadex G-15, eluant water) and lyophilised.

Percentages of amino-acid residues (Tyr : Pro : Gly = 2 : 1 : 1) were confirmed by amino-acid analysis.

Potentiometric Studies.—Formation constants for complexes with H^+ and Cu^{II} were calculated from titration curves carried out at 25 °C using total volumes of 1.5—2.0 cm^3. Alkali was added from a 0.1-cm^3 Hamilton micrometer syringe which had been calibrated by both weight titrations and by titration of standardized materials. Changes in pH were followed using electrodes calibrated in hydrogen-ion concentrations with $HClO_4$. All solutions were of ionic strength 0.10 mol dm^{-3} (KNO_3) with tetrapeptide concentrations of 0.003 mol dm^{-3}. Calculations were made with the aid of the SUPERQUAD computer program.[10] This is a successor to the MINIQUAD program[11] and allows the inclusion of a second ligand and the refinement of the total ligand concentrations. Both the tetrapeptides synthesized contained free acetate as a result of the synthetic technique used and it proved impossible to remove this acetate effectively. However, acetate ions form only weak complexes with Cu^{2+} so that the influence of free acetate on the pH of solutions could be allowed for provided the concentrations were accurately known. SUPERQUAD gave the same



Scheme.

Table 1. Formation constants for H^+ and Cu^{2+} complexes at 25 °C and I = 0.10 mol dm^{-3} (KNO_3), with standard deviations on the last figure given in parentheses

(*a*) H^+ complexes

Ligand (H_3L)	log β_{HL}	log β_{H_2L}	log β_{H_3L}	log β_{H_4L}
Tyr-Pro-Gly-Tyr	10.12(1)	19.87(1)	27.48(1)	30.21(1)
Tyr-Gly-Pro-Tyr	10.16(2)	19.96(1)	27.50(2)	30.52(3)
Tyr-Gly[a] (H_2L)	9.93	17.61	20.77	
Tyr-amide[a] (HL)	9.86	17.33		

Stepwise protonation constants[b]

Ligand (H_2L)	log K_{H_2L}	log K_{H_3L}	log K_{H_4L}
Tyr-Pro-Gly-Tyr	9.75	7.61	2.74
Tyr-Gly-Pro-Tyr	9.80	7.54	3.02

(*b*) Cu^{2+} complexes

	log β values for species					
Ligand (H_2L)	[CuL]	[CuHL]	[CuH_2L]	[$Cu(H_2L)_2$]	[$(CuL)_2$]	[$(CuH_{-1}L)_2$]
Tyr-Pro-Gly-Tyr	8.42(5)	17.73(5)	24.86(3)	48.70(7)		
Tyr-Gly-Pro-Tyr	10.26(9)	19.70(1)	24.36(5)		24.00(5)	3.66(2)
Tyr-Gly[a] (H_2L)	11.41	15.18	24.08			7.26
Tyr-amide[a] (HL)	8.09	14.37				

Stepwise protonation constants

Ligand (H_3L)	log K_{CuHL}	log K_{CuH_2L}	log K_D[c]
Tyr-Pro-Gly-Tyr	9.80	7.54	
Tyr-Gly-Pro-Tyr	9.44	4.66	3.48

[a] Ref. 5. [b] $K_{H_nL} = [H_nL]/[H][H_{n-1}L]$. [c] $K_D = [(CuL)_2]/[CuL]^2$.

concentration ratios for tetrapeptide to acetate over a range of total concentrations of ligand and the calculated formation constants were entirely consistent. The value for the protonation constant for acetate was calculated by iteration (log K = 4.60) and found to be very close to the literature value (4.56).[12] Molar ratios found for tetrapeptide : acetate were 1 : 0.11 for Tyr-Pro-Gly-Tyr and 1 : 0.55 for Tyr-Gly-Pro-Tyr. In all cases duplicate or triplicate titrations were performed at Cu : L ratios of 1 : 2.

Spectroscopic Studies.—Molar ratios of M : L of 1 : 3 were used with metal-ion concentrations of 0.001—0.005 mol dm^{-3}. $Cu(ClO_4)_2\cdot6H_2O$ was the source of Cu^{2+} ions. E.s.r. spectra were recorded on a JEOL JES-ME-3X spectrometer at 120 K and 9.15 GHz. Absorption spectra were recorded on a Beckman UV 5240 spectrophotometer. C.d. spectra were recorded on an automatic JASCO-J-20 spectropolarimeter in the 800—200 nm region. Results are expressed in terms of $\Delta\varepsilon = \varepsilon_l - \varepsilon_r$.

Results and Discussion

Calculated protonation constants and Cu^{2+} complex formation constants for the tetrapeptides (H_3L) studied are given in Table 1, together with literature values for Tyr-Gly (H_2L) and Tyr-

Table 2. Spectroscopic data for Cu^{2+} complexes of Tyr-Pro-Gly-Tyr and Tyr-Gly-Pro-Tyr

pH	Major species	Visible λ/nm (ε/dm^3 mol^{-1} cm^{-1})	C.d. λ/nm ($\varepsilon_l - \varepsilon_r$)	C.d. Assignment	E.s.r. $A_{\parallel}$/G	E.s.r. $g_{\parallel}$	E.s.r. $g_{\perp}$
(*a*) Tyr-Pro-Gly-Tyr							
5.4	[CuH_2L]	720 (60)	693 (−0.38)	$B + E$ (*d–d*)	160	2.294	2.074
			265 (+0.028)	(broad band)			
8.4	[CuHL]	657 (70)	690 (−0.40)	$B + E$ (*d–d*)	152	2.274	2.072
		400 (290)	270 (sh) (+0.3)	NH_2–Cu (c.t.)			
			250 (+0.32)	Intraring			
10.8	[CuL]		670 (−0.24)	$B + E$ (*d–d*)			
			400 (−0.02)	O_{Tyr}^-–Cu (c.t.)			
			298 (−0.12)	NH_2–Cu (c.t.)			
(*b*) Tyr-Gly-Pro-Tyr							
7.2	[CuHL]	630 (120)	572 (−0.14)	$B + E$ (*d–d*)	156	2.240	2.071
			310 (+0.38)	N^-–Cu (c.t.)			
			287 (−0.10)	NH_2–Cu (c.t.)			
			272 (+0.08)	Intraring			
9.6	[$(CuL)_2$]	630 (120)	513 (−0.19)	$B + E$ (*d–d*)	159	2.237	2.065
		392 (196)	360 (+0.03)	O_{Tyr}^-–Cu (c.t.)			
			330 (−0.18)	N^-–Cu (c.t.)			
			300 (+0.06)	NH_2–Cu (c.t.)			
			281 (−0.47)	Intraring			
10.5	[$(CuH_{-1}L)_2$]	590 (sh) (108)	528 (−0.20)	$B + E$ (*d–d*)	168	2.224	2.063
		395 (430)	355 (+0.09)	O_{Tyr}^-–Cu (c.t.)			
			327 (−0.13)	N^-–Cu (c.t.)			
			296 (+0.30)	NH_2–Cu (c.t.)			
			270 (−0.33)	Intraring			

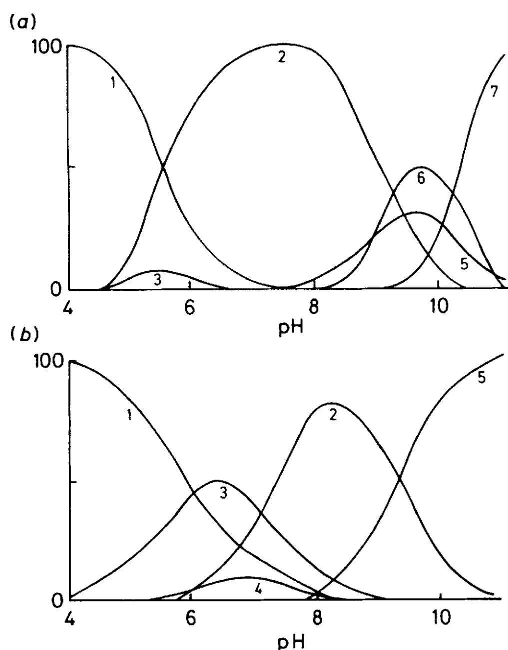


Figure 1. Species distribution curves (%) for 1 : 1 Cu : L mixtures (0.001 mol dm^{-3}) for the tetrapeptides (*a*) Tyr-Gly-Pro-Tyr and (*b*) Tyr-Pro-Gly-Tyr: 1, Cu^{2+}; 2, [CuHL]; 3, [CuH_2L]; 4, [$Cu(H_2L)_2$]; 5, [CuL]; 6, [$(CuL)_2$]; 7, [$(CuH_{-1}L)_2$]

amide (HL). When comparing these values it should be noted that these ligands contain only one phenolic hydroxyl group, not two as in the tetrapeptides. Calculated species distribution curves for 1 : 1 Cu : L mixtures (0.001 mol dm^{-3}) are shown in Figure 1. Spectroscopic data for the complexes are given in Table 2.

Protonation constants are close to those expected from values for comparable peptides. The protonation reactions represented by K_{HL} (*i.e.*, β_{HL}) and K_{H_2L} will be predominantly protonation of phenolate oxygens, K_{H_3L} will be protonation of the terminal $-NH_2$ group, and K_{H_4L} the carboxylate group. As expected, protonation of the terminal Tyr residues is little affected by reversal of the second and third residues of the tetrapeptide chain.

In contrast to the proton co-ordination, Cu^{2+} co-ordination is profoundly affected by the position of the Pro residue. In the second position it promotes the formation of a most unusual [CuHL] species which is yellow-green as a result of phenolate O^-–Cu bonding which begins to take place at the unusually low pH of 6 and which must involve a large chelate ring. With Pro in the third position phenolate–Cu interaction only takes place at higher pH and through dimer formation. Results for the two tetrapeptides will be discussed separately.

Tyr-Pro-Gly-Tyr (H_3L).—The [CuH_2L] species (overall charges are omitted throughout for clarity) has NO co-ordination, bonded through the terminal $-NH_2$ nitrogen and the CO oxygen of the neighbouring peptide linkage, both phenolate oxygens being protonated over the pH in which the species exists. This is confirmed by the absorption spectrum which has a *d–d* band at 720 nm.[5,7] Although the $-NH_2$–Cu charge-transfer (c.t.) region is strongly disturbed by intra-ring transitions, the N^-–Cu c.t. band is absent. The size of the formation constant for the reaction Cu + $H_2L \longrightarrow$ [CuH_2L] (log K = log β_{CuH_2L} − log β_{H_2L} = 24.86 − 19.87 = 4.99) also supports this mode of co-ordination. The bis-complex, [$Cu(H_2L)_2$], forms significantly only in the presence of excess ligand and would be bonded similarly.

The [CuHL] species is the most interesting. It is the major

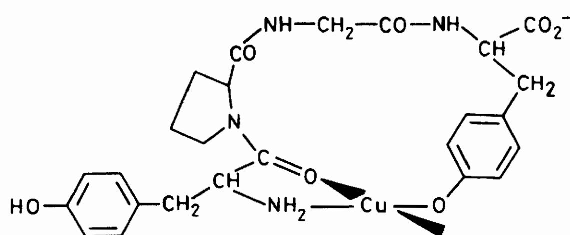


Figure 2. Proposed structure for the [CuHL] species of Tyr-Pro-Gly-Tyr

species over the pH range 7.4—9.6, and its stability {log K(Cu + HL ⟶ [CuHL]) = 7.61} is higher than the comparable value for [CuH_2L] (4.99) but lower than that for the [CuHL] species of Tyr-Gly-Pro-Tyr (9.54). This suggests that the co-ordination involves more than NO but not the NN donor centres normally found in comparable complexes of peptides. In the absorption spectrum the clearest band to emerge with this species is the O_{Tyr}^-–Cu c.t. transition at 400 nm while the (B + E) band at 690 nm observed in the *d–d* region of the c.d. spectrum is typical of a 1N species. All this evidence suggests that the co-ordination of Cu^{2+} in the [CuHL] species consists of bonding to the terminal $-NH_2$ nitrogen, the CO oxygen of the first peptide linkage, and the O_{Tyr}^- of the O-terminal Tyr residue, Tyr(4). This structure is shown in Figure 2 and necessitates a 17-membered chelate ring. This co-ordination scheme is the same as that proposed for Cu–Gly-Pro-Gly-Tyr.[6] The chelate ring is unexpectedly large but is comparable to the 11-membered ring proposed for Gly-Pro-Gly-Gly except that now the chelate ring is to the O_{Tyr}^- of Tyr(4) rather than the peptide-N.[1] From space-filling models it is clear that this species is sterically favourable, the Pro(2) residue is acting as a β-turn in the peptide chain and so forcing the bend required to bring the Tyr(4) oxygen close to the terminal $-NH_2$ group. In addition it is possible that the large chelate ring is stabilised by hydrophobic interaction between the aliphatic ring of –Pro– and the aromatic ring of –Tyr(4).* Since the nitrogen atom of a Pro(2) residue does not possess an ionizable proton it cannot form a typical peptide bond to Cu^{2+}, hence the normal peptide co-ordination sequence cannot be followed and the Pro residue acts as a 'break-point' to Cu^{2+} co-ordination.[1] In this species the O_{Tyr} of the first Tyr residue will be protonated and not co-ordinated to the Cu^{2+}. The terminal carboxylate group is unable to interact as long as the O_{Tyr}^- of Tyr(4) is co-ordinated to the Cu^{2+}.

From the potentiometric studies the [CuHL] species loses a proton above pH 9 to give [CuL]. The stepwise protonation constant (9.80) is very close to those for the protonation of the O_{Tyr}^- atoms of the parent tetrapeptide (10.12 and 9.75). Hence it is reasonable to suppose that this [CuL] complex is the result of deprotonation of the unco-ordinated O_{Tyr} of Tyr(1).

Above pH 9.5 the tetrapeptide undergoes slow hydrolysis, decomposing significantly after 2—3 h. It was possible to collect potentiometric data before decomposition was significant, and to identify the [CuL] species, but spectroscopic data above this pH were not very precise. The band at 400 nm was still observed demonstrating continued involvement of the Tyr side-chain in co-ordination. This band was also present in the c.d. spectra supporting O_{Tyr}^-–Cu bonding. In the *d–d* region of the c.d. spectrum the major band centred around 670 nm supports 1N co-ordination. There is no distinct N^-–Cu c.t. band in the pH 10—10.5 region. The limited spectroscopic data therefore also support a structure similar to that proposed for the [CuHL] species but with the oxygen of Tyr(1) deprotonated. It is unusual for Cu–N^- bonding to be absent in peptide–Cu complexes above pH 10, however space-filling models demonstrate considerable steric hindrance to such species if the co-ordination scheme shown in Figure 2 is retained. In particular the peptide-N of Tyr(4) cannot bond to the Cu^{2+} without breaking the Cu–O_{Tyr}^- bond; hence the unusual co-ordination required for Figure 2 would have an even greater range of existence.

* We would like to thank the referee for making this suggestion.

Tyr-Gly-Pro-Tyr (H_3L).—The ligand may be regarded as comparable to a simple tetrapeptide (HL) with the addition of two ionizable phenolic protons. With this ligand the [CuH_2L] species is a minor species at low pH and comparable to [CuL] with a tetrapeptide such as tetraglycine. Its stability is comparable and it can be assumed that the co-ordination centres are the same, *i.e.* $-NH_2$ and the neighbouring peptide-linkage oxygen. The complex [CuHL] is the major species over the pH range 5—9. Formation of the [CuHL] species may be expressed as Cu + H_2L ⟶ [CuHL] + H, where log $K = -0.26$ (*i.e.* log β_{CuHL} − log β_{H_2L}). This is almost identical to that for the [$CuH_{-1}L$] complex of tetraglycine (−0.30), hence the structures are probably similar with NN co-ordination ($-NH_2$ and the first peptide-N). This is supported by the spectroscopic evidence. The band observed at 630 nm is typical of the energy for *d–d* transitions in Cu complexes with two bound nitrogens and the c.d. spectra also show c.t. transitions at 310 nm (typical of N^-–Cu bonding) and at 287 nm (typical of $-NH_2$–Cu bonding).[7–9] E.s.r. spectra also confirm the presence of just one major species over the pH range 6—8.5. Hence it can be assumed that it is a 2N complex, bonded to the terminal $-NH_2$ group and the peptide-N of the glycine residue.

Pro as the third residue of the chain acts as a break-point to further Cu–peptide-N co-ordination, hence further deprotonation is unlikely to be from a peptide-N.[1] Above pH 7.5 a new band appears in the absorption spectrum at around 400 nm, typical of O_{Tyr}^-–Cu bonding and potentiometric data in this range show the formation of a [CuL] complex and its dimer, [$(CuL)_2$]. Clearest evidence for this dimer comes from the e.s.r. spectra which show a $\Delta m_s = \pm 2$ transition at $g = 4$ when the pH increases above 9. The e.s.r. parameters for the $g = 2$ line of the [$(CuL)_2$] species are close to those observed for the [CuHL] species (see Table 2) suggesting that the complex is still a 2N complex. The deprotonation prompting the dimerisation would therefore be ionization of a Tyr phenolic group. This is supported by the c.d. spectra which show a positive band at 360 nm (O_{Tyr}^-–Cu c.t.) and also bands typical of NH_2–Cu and N^-–Cu bonding around 300 and 330 nm respectively. It is therefore probable that [CuL] is similar to [CuHL] but with a deprotonated Tyr side-chain and the dimer, [$(CuL)_2$], is two [CuL] species linked by O_{Tyr}^-–Cu interaction, most likely from Tyr(1). The potentiometric results are entirely compatible with this structure.

Above pH 10 a deprotonated dimer, [$(CuH_{-1}L)_2$], forms. The c.d. and absorption spectra are virtually unaffected by this deprotonation. Hence deprotonation does not appear to affect the co-ordination sphere and is probably well removed from the co-ordination centres. It may therefore be assumed to be deprotonation of the unco-ordinated tyrosine side-chain, probably Tyr(4) since this is far removed from the Cu^{2+} ion. Potentiometric results also support this model.

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