A Thermodynamic and Spectroscopic Study of the Complexes of the Undecapeptide Substance P, of its N-Terminal Fragment and of Model Pentapeptides containing Two Prolyl Residues with Copper Ions

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Eight pentapeptides have been synthesised which either are models of the N-terminal pentapeptide fragment of Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) or assist in understanding its co-ordinating ability; Gly-Pro-Gly-Pro-Gly, Gly-Pro-Gly-Pro-Glu, Gly-Pro-Gly-Pro-Gln, Gly-Pro-Lys-Pro-Gly, Arg-Pro-Gly-Pro-Gly, Arg-Pro-Lys-Pro-Gln (Substance P_{1-5}), Gly-Pro-Pro-Gly-Gly and Glu-Pro-Pro-Gly-Gly. A potentiometric and spectroscopic study of the complexes formed with H^+ and Cu^{2+} and a potentiometric study of the complexes with Substance P have been made. The results demonstrate the profound effect which the prolyl residue can have, when incorporated in a peptide chain, on the formation of copper(II)–peptide complexes. It acts as a break-point to co-ordination and encourages the formation of folded peptide chains, through β turns, resulting in large, but very stable, chelate rings. The co-ordination behaviour of Substance P is almost identical to that of the N-terminal fragment, Substance P_{1-5}, with chelation through the N-terminal amino N and the ε-amino N of the Lys residue to form a large chelate ring of high stability, the peptide being forced into a bent conformation by the prolyl residue. With Substance P and its analogues, bonding between Cu^{II} and deprotonated peptide nitrogens is absent below pH 10 but with the pentapeptides containing the Pro-Pro unit co-ordination to the peptide N of a Gly residue takes place surprisingly easily (starting at pH 7) to form a large chelate ring.

Substance P is a peptide containing 11 amino acid residues in the sequences Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2.†[2] It is a member of the group of tachykinins, a family of biogenic peptides sharing the common C-terminal sequence Phe-Xaa-Gly-Leu-Met-NH_2 (where Xaa is an amino acid residue) and to some extent common biological functions.[3] It is present in various parts of the central nervous system and in the gastrointestinal tract of mammals. Its actions are believed to include the transmission of pain information, vasodilation, smooth muscle contraction, modulation of signal transmissions in the striato-nigral pathway, evocation of local inflammatory responses and possibly also action as a messenger between the immune system and the brain.[4,5]

At the present time three classes of tachykinin receptors are recognised, conforming to three known mammalian tachykinins: Substance P (SP), and neurokinin A and B. Binding to these receptors is quite selective, in contrast to the cross-reactivity observed for non-mammalian tachykinin-like peptides, which still share a common C-terminal sequence.[6] The C-terminal part of the SP molecule is recognised by the receptor, the SP_{6-11} fragment being a minimal active species in most tests, especially outside the central nervous system.[4,7] However some neuronal test systems show the absolute requirement for the N-terminal part[8,9] and enzymatic cleavage of SP in biological systems results, among others, in the liberation of the peptide fragments SP_{1-4}, SP_{1-7}, (pyro-Glu^5)-SP_{5-11} and SP_{8-11}.[4]

Numerous studies have been performed to elucidate the relationship between the structure and activity of SP. As a result of the incomplete characterisation of a receptor site several applied approaches have been followed, including the testing of chemically modified SP analogues in various receptor systems,[10,11] conformational studies of the active forms in various solvents and theoretical.[12–14] These studies suggest that the active conformation of SP contains an α-helix beginning at about the fourth residue and including most of the C-terminal region. The N-terminal tetrapeptide of SP, apart from its distinct biological function, shows a unique solution feature. In aqueous solution there exists an equilibrium between two forms, differing by a *cis* or *trans* configuration of the Lys-Pro peptide bond, the *cis* species being stabilised by ionic interaction of the lateral Lys-NH_2 group with the terminal carboxyl.[15,16]

Substance P is able to release histamine from rat peritoneal mast cells in a concentration-dependent manner[17] and it is known that histamine release in the early phase of the inflammatory response coincides with an increase in the copper concentration in blood plasma.[3,16] Copper activation of various neuropeptides, such as enkephalins,[18] Thyrotropin Releasing Factor (TRF)[19] and Luteinizing hormone Releasing hormone (LHRH),[20] has been discussed. Taking this into account, it is appropriate to consider a possible link between the two above physiological processes although there

† Abbreviations used for amino acid residues are those recommended by IUPAC-IUB.[1]

is no known link between the activity of Substance P and copper.

We have undertaken an investigation of the co-ordination abilities of the SP fragments and their analogues towards copper(II) ions, with special attention to the role of the proline residues in the conformation of the ligand chain since it is known that this residue can act as a 'break point' to metal ion co-ordination.[21–24] Proline, as the only naturally occurring amino acid containing a secondary nitrogen atom, is unable to form a N^-–Cu bond and therefore it induces a break in the normal mode of co-ordination achieved by regular peptides such as tetraalanine.[25] In the latter case co-ordination consists of the deprotonation and binding of the terminal amino group at low pH, followed by a stepwise deprotonation and binding of successive peptide nitrogens as the pH is raised until the square-planar geometry of 4N donors is produced with the tetrapeptide held in a tight, circular conformation. The presence of the Pro residue in the chain leads to very specific modes of binding including the formation of dimers or of large chelate rings.[21,22] Since SP contains two Pro residues (in the second and fourth positions) these could have a profound effect on its conformation and ability to co-ordinate to Cu^{II}.

In this paper we report the synthesis of five pentapeptides which are analogues of the SP N-terminal sequence: *i.e.* Gly-Pro-Gly-Pro-Gly, Gly-Pro-Gly-Pro-Glu, Gly-Pro-Gly-Pro-Gln, Gly-Pro-Lys-Pro-Gly and Arg-Pro-Gly-Pro-Gly and of the SP_{1-5} fragment, Arg-Pro-Lys-Pro-Gln, together with two pentapeptides which have the two Pro residues adjacent in positions 2 and 3 rather than separated by Gly or Lys: Gly-Pro-Pro-Gly-Gly and Glu-Pro-Pro-Gly-Gly. Complexes formed by these pentapeptides and by the undecapeptide, Substance P, have been studied potentiometrically and spectroscopically using electronic absorption, circular dichroism (CD) and electron spin resonance (ESR) spectroscopy. A preliminary account of the results has been presented recently.[26]

Experimental

Peptide Syntheses.—The pentapeptides were synthesised by standard liquid-phase methods using, as starting materials: Bu^tOCO-Arg(NO_2), Bu^tOCO-Gly, Bu^tOCO-Lys(Z), Bu^tOCO-Gln, Bu^tOCO-Glu-OCH_2Ph, HCl·Gly-OMe, HCl·Pro-OMe and HCl·Glu(OCH_2Ph)$_2$.

Glutamine benzyl ester hydrochloride. To Bu^tOCO-Gln (3 g) in diethyl ether, with a few drops of ethanol, was added slowly an ethereal solution of freshly prepared diazotoluene. Addition was continued until the vigorous reaction subsided and the reddish brown colour of the diazotoluene persisted. After evaporation of the solvent, the Bu^tOCO group was removed using 2 mol dm^3 HCL in ethanoic acid. Glutamine benzyl ester hydrochloride was obtained as a white powder by addition of diethyl ether (90%).

General procedure. Coupling and deprotection steps were followed by TLC (Merck G60, eluent $CHCl_3$–MeOH–$MeCO_2H$ 85:15:5). Free pentapeptides were purified by chromatography on Whatman 3 (eluent butanol–pyridine–acetic acid–water 30:20:6:24).

Coupling steps. C-Protected derivatives [in $CHCl_3$ or dimethylformamide (dmf) solution] were neutralised with triethylamine. The Bu^tOCO-amino acid was dissolved in this solution (at 0 °C) followed by addition of dicyclohexylcarbodiimide (dcci) (Merck) and 1-hydroxybenzotriazole (Aldrich) in 10% excess and the solutions kept at 0 °C for 3 h. After standing overnight at room temperature, the dicyclohexylurea was filtered and the $CHCl_3$ solution washed with $NaHCO_3$ solution (5%, twice), water, HCl (1%, twice) and again with water.

Deprotecting steps. After drying over $MgSO_4$ and evaporation of the solvent, protecting groups were removed as follows.

Bu^tOCO. The protected derivative was dissolved in a few cm^3 of dioxane and a cold solution (4 °C) of 4 mol dm^{-3} HCl in dioxane added. After 30 min TLC plates showed complete cleavage. The solvent was removed by evaporation and the oily material triturated with diethyl ether to give the hydrochloride of the C-protected (or free) peptide.

Methyl ester. The protected derivative was dissolved in methanol, cooled to 0 °C and 1.1 equivalents of aqueous NaOH added. When cleavage was complete, 10% HCl was added to pH 3 and the methanol evaporated. The N-protected peptide was extracted with ethyl acetate (three times) and dried over $MgSO_4$. It was recrystallised from ethyl acetate–hexane.

Benzyl, nitro and benzyloxycarbonyl (Z) groups. The protected peptides, in ethanoic acid solution, were hydrogenated using 10% Pd on charcoal (100 mg catalyst per 0.002 mol peptide). The extent of cleavage was followed by uptake of H_2 and by chromatography on Whatman 3.

As an example, the synthesis of Arg-Pro-Lys-Pro-Gln (Substance P_{1-5}) is outlined in Scheme 1.

Purification of Pentapeptides.—The chloride ion of the cationic form was exchanged using AG1X8 (Bio-Rad) in the acetate form. The resulting zwitterionic peptides were purified by gel filtration (Sephadex G-15; eluent water) followed by freeze drying. All the pentapeptides gave a single spot by paper chromatography.

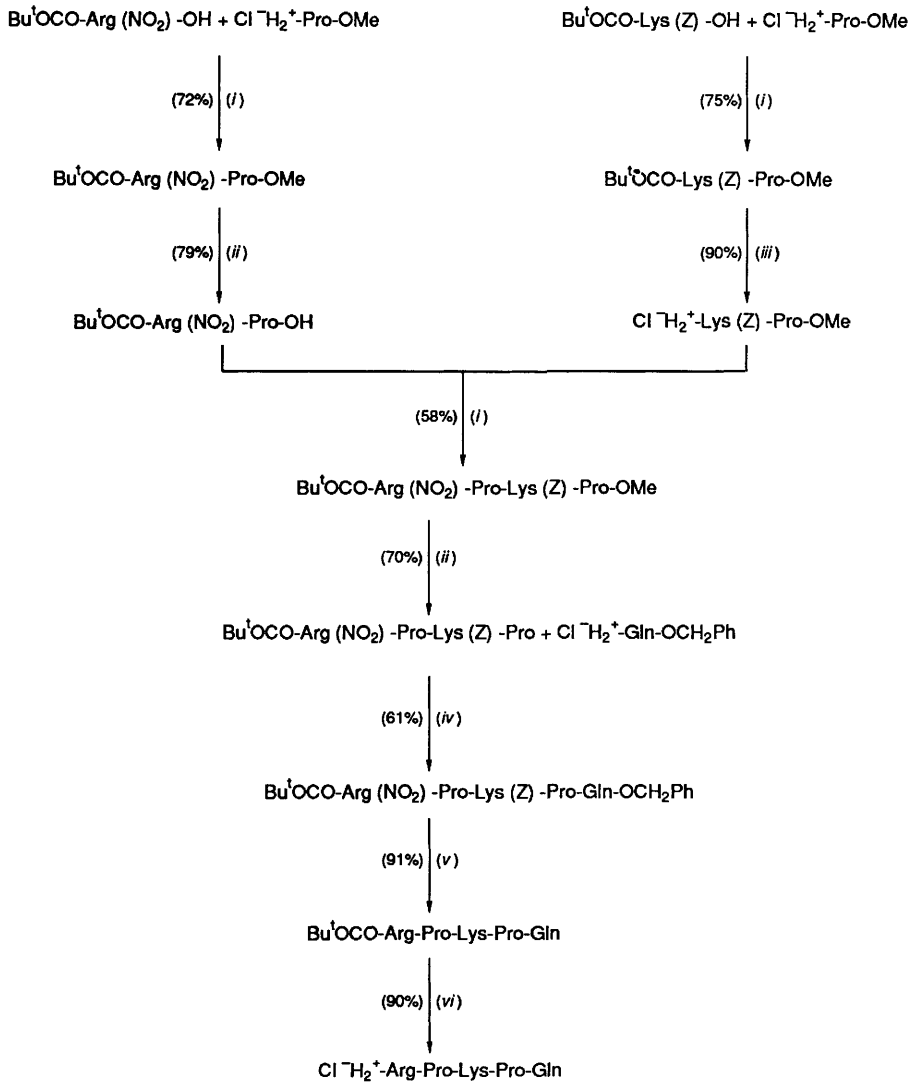
The absence of acetate ions in the products was confirmed by 400 MHz NMR spectroscopy and purity of the peptides was checked by amino acid analysis, elemental analysis (where sufficient samples were available) and fast atom bombardment (FAB) mass spectrometry. The pentapeptides containing the Gln residue could not be checked by amino acid analysis because the amido group of the Gln side chain was removed during acid cleavage of the peptide.

Substance P was a commercial sample (Bachem), provided as the acetate salt. The ratio of Substance P to acetate was determined by potentiometric titration and the ratio checked in each titration performed. A ratio of 1:2.246($\pm$0.009) was found over seven titrations. The purity of all the ligands studied was confirmed by pH-metric titration. The calculation of protonation constants gave highly consistent results with low standard deviations (better than $1\sigma = 0.01$) and random distributions of residuals, without any general trends, reflected in low values for χ^2. Analytical results are given in Table 1.

Potentiometric studies.—Stability constants for complexes of H^+ and Cu^{2+} were calculated from titration curves obtained using total volumes of about 1.5 cm^3. Alkali was added from a 0.1 cm^3 micrometer syringe which had been calibrated by both weight titration and the titration of standardised materials. Experimental details were: peptide concentration 0.003 mol dm^{-3}; copper concentration 0.001–0.0028 mol dm^{-3}; ionic strength 0.10 mol dm^{-3} (KNO_3); pH range for complexation 4–9.7; method, pH-metric titration, calibrated in concentrations using $HClO_4$;[27] number of titrations three per ligand; temperature 25 °C; method of calculation SUPERQUAD.[28]

Calculations were made with the aid of the SUPERQUAD computer program which allows for the refinement of total ligand concentrations and was able to confirm the purity of the peptides synthesised and, in particular, the absence of acetate. The commercial sample of Substance P contained an excess of acetate ions, which could be included in the SUPERQUAD calculations once its concentration had been measured by pH-metric titration. Standard deviations (1σ) quoted were computed by SUPERQUAD and refer to random errors only. They give, however, a good indication of the importance of the particular species in the equilibrium.

Spectroscopic Studies.—Solutions were of similar concentrations to those used in the potentiometric studies, using $Cu(ClO_4)_2 \cdot 6H_2O$ as the source of copper(II) ions. Absorption spectra were recorded on a Beckman UV 5240 spectrometer and CD spectra on automatic recording spectropolarimeters (JASCO-J-20 and Yvon-Jobin Mark III dichrograph) in the



Scheme 1 (*i*) (*a*) NEt_3, (*b*) Bu^{t}OH–dcci in $CHCl_3$; (*ii*) NaOH in MeOH; (*iii*) 4 mol dm^{-3} HCl in dioxane; (*iv*) (*a*) NEt_3, (*b*) Bu^{t}OH–dcci in dmf; (*v*) 10% Pd/C in $MeCO_2H$; (*vi*) 2 mol dm^{-3} HCl in $MeCO_2H$

800–200 nm region. ESR spectra were obtained on a JEOL JES-Me-3X spectrometer at 130 K and at 9.13 GHz, FAB mass spectra on a VG AutoSpec spectrometer.

Results

Stability constants of hydrogen-ion complexes are given in Table 2. Two of the pentapeptides synthesised, as well as SP itself, contain an Arg residue. The guanidine side-chain of this residue does not normally co-ordinate to Cu^{II} or similar metals.[29] Protonation and complex formation by this side-chain was therefore not included in the equilibrium calculations. All ligands are assumed to have the empirical formula HL, charges are omitted for clarity. Stability constants of the complexes with Cu^{II} are also given in Table 2 and the species distribution curves for selected pentapeptides and for Substance P, calculated for 0.001 mol dm^{-3}, are shown in Fig. 1. Spectroscopic data for the copper(II) complexes are shown in Table 3. With all the ligands in the presence of Cu^{II}, slow precipitation was observed above pH 10.

Discussion

For the six pentapeptides without a Lys residue the protonation represented by β_{HL} corresponds to protonation of the α-amino-nitrogen. The values for Gly-Pro-Gly-Pro-Gly, Gly-Pro-Gly-Pro-Gln and Gly-Pro-Pro-Gly-Gly are very close to that for Gly-Pro-Gly-Gly (log β = 8.25)[21] with Gly-Pro-Gly-Pro-Glu a little larger. The constant for Glu-Pro-Pro-Gly-Gly is somewhat lower as a result of the inductive effect of the carboxyl group of the Glu side-chain. With Arg-Pro-Gly-Pro-Gly the constant is significantly smaller as a result of the positive charge on the Arg residue. In the case of peptides containing a Lys residue (including SP itself) protonation of the α-amino and ε-amino-nitrogens will overlap somewhat but protonation of the ε-amino group of the Lys would be expected to occur at higher pH making it the greater contributor to the first macroconstant.[29] The second protonation constant of these peptides would then be the macroconstant for the second amino protonation, mostly the α-NH_2. The other protonation constants refer to carboxyl protonations. Substance P itself has no carboxyl groups, hence it shows only two protonation constants with values comparable to those for Arg-Pro-Lys-Pro-Gln.

Copper(II) *Complexes*.—A number of peptides, particularly when they contain the Lys residue, are able to form dimeric complexes with Cu^{II}. In the systems studied there was no evidence of dimerisation in the ESR spectra and models used to fit the potentiometric data which included dimeric species were either rejected or gave results which were less satisfactory statistically than those calculated for simpler systems which omitted dimers. Spectroscopic results indicate that the major

Table 1 Analytical data for the pentapeptides synthesised

Amino acid analysis

	Gly	Pro	Others
Gly-Pro-Gly-Pro-Gly	1.0	0.61	
Gly-Pro-Gly-Pro-Glu	1.0	0.93	Glu 0.51
Arg-Pro-Gly-Pro-Gly	1.0	1.01	Arg 0.48
Gly-Pro-Lys-Pro-Gly	1.0	1.02	Lys 0.46
Gly-Pro-Pro-Gly-Gly	1.0	0.64	
Glu-Pro-Pro-Gly-Gly	1.0	0.97	Glu 0.52

FAB-mass spectroscopy *

	Major peak	Minor peaks
Gly-Pro-Gly-Pro-Gly	383.4 + 1 (H)	383 + 23 (Na) and 383 + 133 (Cs)
Gly-Pro-Gly-Pro-Glu	455.3 + 1	455 + 23 and 455 + 134 (Cs + H)
Gly-Pro-Gly-Pro-Gln	454.3 + 1	454 + 23, 454 + 133
Gly-Pro-Lys-Pro-Gly	454.4 + 1	454 + 23, 454 + 133 and 454 + 156 (Cs + Na)
Arg-Pro-Lys-Pro-Gln	624.5 + 1	624 + 23
Gly-Pro-Pro-Gly-Gly	383.3 + 1	383 + 23, 383 + 133
Glu-Pro-Pro-Gly-Gly	455.3 + 1	455 + 23, 455 + 133

Elemental analysis (%)

	Found				Required		
	C	H	N		C	H	N
Gly-Pro-Gly-Pro-Gly	46.8	6.8	16.85	$C_{16}H_{25}N_5O_6 \cdot 1.5H_2O$	46.8	6.85	17.05
Gly-Pro-Gly-Pro-Glu	45.65	6.7	14.05	$C_{19}H_{29}N_5O_8 \cdot 2.5H_2O$	45.6	6.85	14.0
Gly-Pro-Gly-Pro-Gln	45.05	6.85	16.3	$C_{19}H_{30}N_6O_7 \cdot 3H_2O$	44.9	7.1	16.5
Gly-Pro-Pro-Gly-Gly	46.15	6.7	16.5	$C_{16}H_{25}N_5O_6 \cdot 2H_2O$	45.85	6.9	16.65
Glu-Pro-Pro-Gly-Gly	46.2	6.5	13.8	$C_{19}H_{29}N_5O_8 \cdot 2H_2O$	46.4	6.75	14.1

* m/z values; matrix = *m*-nitrobenzyl alcohol.

Table 2 Stability constants (log β values) for complexes of H^+ and Cu^{2+} at 25 °C and I = 0.10 mol dm^{-3} (KNO_3)

	Proton complexes (estimated standard deviations 0.01)			Copper(II) complexes			
	HL	H_2L	H_3L	Cu(HL)	CuL	CuL_2	$\hat{C}uH_{-1}L$
Gly-Pro-Gly-Pro-Gly ($C_{16}H_{25}N_5O_6$)	8.36	11.67			5.88(2)	10.60(2)	
Gly-Pro-Gly-Pro-Glu ($C_{19}H_{29}N_5O_8$)	8.53	12.93	15.39		6.57(5)	10.54(9)	
Gly-Pro-Gly-Pro-Gln ($C_{19}H_{30}N_6O_7$)	8.29	11.51			5.73(1)	10.16(2)	
Arg-Pro-Gly-Pro-Gly ($C_{20}H_{34}N_8O_6$)	7.47	10.86			4.83(1)	8.96(1)	
Gly-Pro-Lys-Pro-Gly ($C_{20}H_{34}N_6O_6$)	10.24	18.36	21.77	15.45(1)	8.99(1)		
Arg-Pro-Lys-Pro-Gln ($C_{27}H_{48}N_{10}O_7$)	9.85	17.12	21.12	14.30(4)	7.84(2)		
Substance P ($C_{63}H_{98}N_{18}O_{13}S$)	10.06	17.28		14.35(9)	7.73(3)		
Gly-Pro-Pro-Gly-Gly ($C_{16}H_{25}N_5O_6$)	8.30	11.58			5.38(3)		−1.36(3)
Glu-Pro-Pro-Gly-Gly ($C_{19}H_{29}N_5O_8$)	7.97	12.17	15.26		5.18(1)		−1.82(2)

co-ordination site in all systems studied is the N-terminal nitrogen donor. At pH <9.5 two distinct copper(II) complexes are observable using spectroscopic techniques. The energy of the d–d transitions as well as the charge-transfer bands show these to be species with 1N and 2N co-ordination respectively. The pentapeptides Gly-Pro-Pro-Gly-Gly and Glu-Pro-Pro-Gly-Gly were the only ones which showed spectroscopic evidence for co-ordination between copper and amide nitrogen atoms, with characteristic charge-transfer bands in the CD spectra in the 315–325 nm region (see Table 3). With all the other peptides either one or two N-terminal amino nitrogens were involved in the metal-ion binding but the peptides containing the Lys residue formed only mono complexes [Cu(HL) and CuL] while the others formed mono and bis complexes. Hence, in terms of complex formation with copper, the peptides studied can be divided into three significantly different groups depending on the amino acid sequence of residues 2–4, namely Pro-Gly-Pro, Pro-Lys-Pro and Pro-Pro-Gly. Complexes of the three groups will therefore be considered separately.

Potentiometric studies of the pentapeptides containing the Pro-Gly-Pro sequence show the 1N and 2N species to be CuL and CuL_2 respectively with co-ordination through the amino-N and the carbonyl oxygen of the neighbouring peptide linkage. With Gly-Pro-Gly-Pro-Gly and Gly-Pro-Gly-Pro-Gln the values for log β_{CuL} are similar to that for Gly-Pro-Gly-Gly while the value for Arg-Pro-Gly-Pro-Gly is rather lower as a result of the positive charge on the Arg residue. With Gly-Pro-Gly-Pro-Glu, however, the CuL complex is of more significance than with the others, the stability constant being higher by about 0.7 log units (see Table 2). This stabilisation must result from co-ordination to the lateral carboxyl group of the Glu residue since this is the only difference between this ligand and the Gly^5 analogue; this was difficult to accept initially as the resulting chelate ring would be 18- or 19-membered (depending on whether the carbonyl O of the N-terminal Gly residue is co-ordinated). Independent support for such bonding was obtained from the CD spectrum which had a very intense, broad band at 250 nm. This energy is on the border of the range accepted for NH_2–Cu charge-transfer transitions but is typical of CO_2^-–Cu bands.[30] If this is the case, it is yet another striking example of the influence of the prolyl residue on the conformation of peptide molecules. The effect of this increase in stability resulting from the inclusion of Glu as the O-terminal

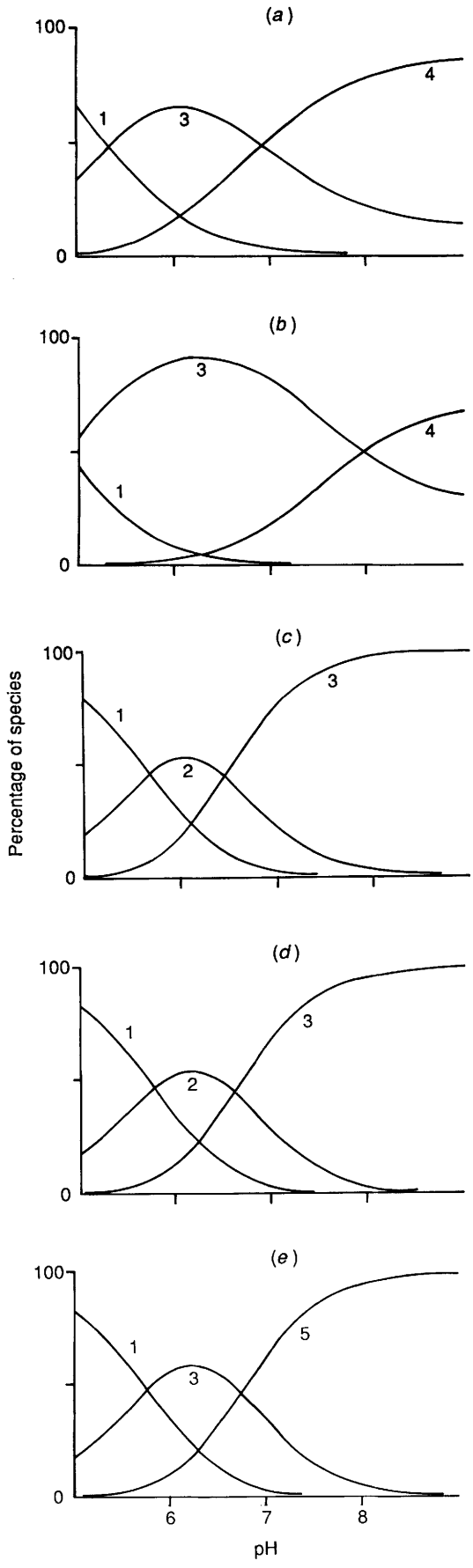


Fig. 1 Species distribution curves for complexes of 1:2 mixtures of Cu^{II} (0.001 mol dm^{-3}) with (*a*) Gly-Pro-Gly-Pro-Gly, (*b*) Gly-Pro-Gly-Pro-Glu, (*c*) Arg-Pro-Lys-Pro-Gln (SP_{1-5}), (*d*) Substance P, (*e*) Gly-Pro-Pro-Gly-Gly. Curves: 1, Cu^{2+}; 2, Cu(HL); 3, CuL; 4, CuL_2; 5, $CuH_{-1}L$

residue is evident from a comparison of the species distribution curves shown in Fig. 1(*a*) and (*b*).

There was no spectroscopic evidence for bonding between peptide nitrogens and Cu^{II} (*e.g.* no CD charge-transfer bands in the 300–330 nm region) and no potentiometric evidence for peptide-nitrogen deprotonation. Values calculated for log β (CuL_2) for these peptides also support the suggestion from spectroscopy of 2N co-ordination through the amino nitrogens of two ligand molecules.

The second group of peptides (those containing the Lys^3 residue, including SP itself) gave potentiometric models which are very similar to one another but are distinctly different from the other ligands studied. Spectroscopic results showed 1N and 2N complexes without Cu–N^- (peptide) co-ordination. The combined potentiometric and spectroscopic results demonstrate that with Gly-Pro-Lys-Pro-Gly and Arg-Pro-Lys-Pro-Gln (SP_{1-5}) the Cu(HL) species would be comparable to the CuL species with the other peptides, but with the ε-amino group of the Lys residue protonated making it a 1N complex. Values for log $\beta_{Cu(HL)}$ corrected for this protonation (*i.e.* log $\beta_{Cu(HL)}$ − log β_{HL}) are entirely compatible with this suggestion. The species CuL, shown by spectroscopic studies to be a 2N complex, would then involve chelation of a second nitrogen donor from the side-chain amino group of the Lys residue to form a large chelate ring (13 or 14 membered, depending on whether the carbonyl O of the first Gly residue is co-ordinated). Again this chelation would be encouraged by the β turn of the Pro residue [comparable to similar chelation found with $(Gly\text{-}Gly\text{-}Pro\text{-}Lys)_2$].[21] It is also supported by an X-ray study of the Cu^{II}-Lys-Tyr complex which suggests bonding from the lateral Lys-NH_2 group.[31] Additional supporting evidence for co-ordination of the lateral Lys amino nitrogen comes from the ESR spectra for the 2N complexes which show a small but systematic trend with complexes of ligands containing the Lys^3 residue having slightly larger values for $A_{\parallel}$ and marginally smaller values for $g_{\parallel}$ than the others. This could be caused by the more basic second nitrogen atom, as *e.g.* in the 2N complex with N(amino),N(amide) bonding (*cf.* Pro-Pro peptides in Table 3) and also by a more symmetrical surrounding to the metal ion which could be a result of the high flexibility of the long Lys side-chain. What is more, there was no evidence for normal deprotonation of the ε-amino nitrogen of the lysyl side-chain. In the absence of co-ordination this would be expected to occur above pH 9. Substance P itself behaves in an almost identical fashion to Arg-Pro-Lys-Pro-Gln (SP_{1-5}), forming the same complexed species with almost the same stability constants. This is demonstrated by the almost identical species distribution curves shown in Fig. 1(*c*) and (*d*). It is therefore reasonable to assume that co-ordination will be the same involving 2N (α- and ε-NH_2) co-ordination without Cu–N^- (peptide) bonding but with the peptide backbone locked in a bent conformation. Hence, as a ligand to Cu^{II}, Substance P behaves in an almost identical fashion to its N-terminal pentapeptide fragment.

The 1N and 2N complexes formed by the third group of pentapeptides containing the Pro-Pro-Gly unit were shown by both potentiometry and spectroscopy to be the CuL and $CuH_{-1}L$ species. The CuL species of Glu-Pro-Pro-Gly-Gly is a little weaker than with Gly-Pro-Pro-Gly-Gly suggesting no significant co-ordination through the lateral carboxyl group of the Glu residue. The deprotonated complexes, however, must involve chelation through the terminal amino N (and probably the neighbouring carbonyl O) and a deprotonated peptide N of either the Gly^4 or Gly^5 residues, with the formation of a large chelate ring of either 10 or 13 atoms, assuming additional co-ordination through the carbonyl O of the N-terminal Gly residue.

The stability of this large ring is unexpectedly high, being only about an order of magnitude less than that for the $CuH_{-1}L$ complex of tetraglycine (log $\beta = -0.4$)[30] which forms a stable five-membered chelate ring. It is more stable by an order of magnitude than the corresponding complex with Gly-Pro-Gly-

Table 3 Spectroscopic data for copper(II) complexes with Substance P_{1-5} and its 'model' peptapeptides

Species		Visible λ_{max}[a]/nm	CD λ_{max}[b]/nm	ESR $10^4A_{\parallel}$[c]/cm^{-1}	ESR $g_{\parallel}$
Gly-Pro-Gly-Pro-Glu					
CuL	(1N)	725(49)	756(−0.02)[c] 250 (+0.6)[d]	160	2.325
CuL_2	(2N)	692(63)	695(−0.25)[c] 250(+0.42)[d,e]	160	2.282
Gly-Pro-Gly-Pro-Gln					
CuL	(1N)	732(35)	750(−0.03)[c] 253(+0.08)[d]	159	2.332
CuL_2	(2N)	695(50)	718(−0.10)[c] 260(+0.04)[d]	169	2.277
Arg-Pro-Gly-Pro-Gly					
CuL	(1N)	702(41)	706(−0.09)[c]		
CuL_2	(2N)	660(61)	682(−0.08)[c] 262(+0.19)[d]		
Gly-Pro-Lys-Pro-Gly					
Cu(HL)	(1N)	720(29)	753(−0.09)[c]	159	2.333
CuL	(2N)	662(56)	722(−0.15)[c] 258(+0.41)[d]	175	2.269
Arg-Pro-Lys-Pro-Gln					
Cu(HL)	(1N)		>740(−0.19)[c] 260(−0.2)[d]		
CuL	(2N)		715(−0.14)[c] 270(−0.13)[d]	177	2.270
Gly-Pro-Pro-Gly-Gly					
CuL	(1N)	750(30)	730 (weak) 260(−0.04)[d]	160	2.330
$CuH_{-1}L$	(2N)	672(55)	645(+0.16)[c] 315(−0.18)[f] 270(+0.20)[d] 228(−1.90)[g]	167	2.276
Glu-Pro-Pro-Gly-Gly					
CuL	(1N)	745(40)	750(−0.06)[c] 260(−0.18)[d] 226(+0.67)[g]	171	2.310
$CuH_{-1}L$	(2N)	680(68)	680(+0.08)[c] 325(−0.07)[f] 250(−0.45)[d] 227(+0.69)[g]	180	2.269

[a] Approximate absorption coefficients (ε/dm^3 mol^{-1} cm^{-1}) in parentheses. [b] Δε in parentheses (dm^3 mol^{-1} cm^{-1}). [c] d–d Transition. [d] NH_2–Cu Charge-transfer transition. [e] CO_2^-–Cu Charge-transfer transition. [f] N^-–Cu Charge-transfer transition. [g] Intraligand transition.

Gly (log β = −2.5[21]) and a corresponding chelated complex with Gly-Pro-Gly-Pro-Gly could not be detected. This suggests that the peptide N of a Gly^3 residue in the Gly-Pro-Gly unit cannot chelate effectively and that the chelate ring in Gly-Pro-Gly-Gly must be to the peptide N of Gly^4, as suggested by space-filling models and by the CD spectra of the $CuH_{-1}L$ complex of Gly-Pro-Gly-Phe.[32] Models show that the presence of a Pro-Pro pair creates a very favourable conformation for the formation of a large chelate ring spanning Gly^1 to Gly^4 in good agreement with the unexpectedly high stability found. This is demonstrated in the species distribution curves for the complexes of Gly-Pro-Pro-Gly-Gly shown in Fig. 1(*e*).

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