Potentiometric and Spectroscopic Studies on Copper(II) and Zinc(II) Complexes of Peptides containing Bis(imidazolyl) Ligands

Katalin Várnagy,[a] Imre Sóvágó,[a] Károly Ágoston,[a] Zsuzsanna Likó,[b] Helga Süli-Vargha,[b] Daniele Sanna[c] and Giovanni Micera[c]

[a] *Department of Inorganic and Analytical Chemistry, Lajos Kossuth University, H-4010 Debrecen, Hungary*
[b] *Research Group of Peptide Chemistry, Hungarian Academy of Sciences, H-1518 Budapest 112, PO Box 32, Hungary*
[c] *Department of Chemistry, University of Sassari, Via Vienna 2, I-07100 Sassari, Italy*

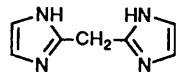
Copper- and zinc-(II) complexes of various bis(imidazolyl) ligands have been studied by potentiometric, visible and EPR spectroscopic methods. The ligands included bis(imidazol-2-yl)methane (CH_2R_2), bis-(imidazol-2-yl)methylamine (R_2CHNH_2) and 3,3-[bis(imidazol-2-yl)]propionic acid ($R_2CHCH_2CO_2H$) and peptides in which the bis(imidazolyl) groups are coupled at the C-terminus, MeCO-Pro-Leu-Gly-$NHCHR_2$ and Bu^tOCO-Pro-His-Gly-$NHCHR_2$, or the N-terminus, R_2CHCH_2CO-Ile-Ala-Gly-OEt and R_2CHCH_2CO-Ile-His-Gly-OEt (where R = imidazol-2-yl). The data revealed that stable mono- and bis-(ligand) complexes are formed with all ligands and the imidazole nitrogens are the main metal binding sites. Tridentate co-ordination of R_2CHNH_2 was concluded to exist in the equimolar solutions of copper(II) and R_2CHNH_2, which results in the formation of a dinuclear mixed-hydroxo complex with imidazole bridging. Deprotonation of the co-ordinated water molecules was also observed around the physiological pH range in the zinc(II)–R_2CHNH_2 complex. The involvement of the side-chain imidazole residues of the peptides Bu^tOCO-Pro-His-Gly-$NHCHR_2$ and especially R_2CHCH_2CO-Ile-His-Gly-OEt in co-ordination has also been demonstrated.

Imidazole nitrogen donor atoms of histidyl residues are the most common binding sites in various metalloenzymes. For example, in carbonic anhydrase zinc(II) ions are co-ordinated by three imidazole rings, in carboxypeptidase by two imidazole rings and a carboxylate anion, while in the blue copper proteins the copper(II) ions are co-ordinated by two imidazole nitrogen and two sulfur atoms of cysteine and methionine. In these metalloproteins the three-dimensional structures of the macromolecules facilitate the co-ordination of metal ions by independent side-chain residues. Therefore, ligands containing two or more imidazole rings linked *via* aliphatic carbon chains can potentially mimic the binding sites and catalytic activities of the enzymes.

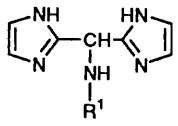
Protonation equilibria and complex formation processes of bis(imidazol-4-yl)methane with several metal ions were first studied by Drey and Fruton.[1,2] A series of polyimidazole ligands including bis(imidazol-2-yl)methane (CH_2R_2) and 3,3-[bis(imidazol-2-yl)]propionic acid ($R_2CHCH_2CO_2H$) (where R = imidazol-2-yl) were prepared by Tang *et al.*[3] and complex formation with copper-, nickel-, cobalt- and zinc-(II) ions has also been studied. The equilibrium studies on the copper(II)–CH_2R_2 system were performed by Mohan.[4] It is clear from this work that bis(imidazolyl) ligands form very stable mono- and bis-(ligand) complexes with divalent transition metal ions *via* the co-ordination of imidazole nitrogens in six-membered chelate rings. The relative stability of the complexes is generally higher than those of the corresponding diamines (*e.g.* propane-1,3-diamine) owing to the π-acceptor properties of imidazole rings.

As a result of the remarkable stability of metal complexes of these ligands another advantage of polyimidazoles is that they may serve as general zinc-enzyme inhibitors. Moreover, specific enzyme inhibitors may be obtained by attaching bis(imidazolyl) ligands to the preferred peptide sequence for the enzyme cleavage. Inhibitors of the collagenase enzyme have already been prepared from peptides containing bidentate hydroxylamine or monodentate thiol groups as binding sites for zinc(II).[5] Here we report the results of combined potentiometric and spectroscopic studies on the copper- and zinc-(II) complexes of peptide molecules containing bis(imidazolyl) ligands which are aimed at inhibiting collagenase activity. The peptides involved in this study are the fragments (and their derivatives) of the specific sequence -Pro-Leu-Gly-Ile-Ala-Gly- of collagen cleaved by the vertebrate collagenases at the glycyl–isoleucine bond. The use of two different bis(imidazolyl)-containing agents, such as bis(imidazol-2-yl)methylamine (R_2CHNH_2) and 3,3-[bis-(imidazol-2-yl)]propionic acid ($R_2CHCH_2CO_2H$) (where R = imidazol-2-yl) made it possible to prepare derivatives of peptides containing the bis(imidazol-2-yl) residues either on their C- or their N-termini. For the sake of the reliable comparison of the complex-forming capabilities of various ligands, the copper- and zinc-(II) complexes of the simple bis(imidazolyl) containing compounds (CH_2R_2, R_2CHNH_2 and $R_2CHCH_2CO_2H$) were also studied.

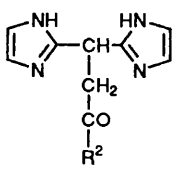
Imidazole has two nitrogen atoms and only one of them was considered as a metal binding site in the previous studies on bis(imidazolyl) ligands. Ionization of the pyrrole-type nitrogen N^1H occurs in very basic solutions (pH > 14), but it has been well demonstrated that certain metal ions [*e.g.* palladium- and copper-(II)] can induce deprotonation or even co-ordination of N^1 in complexes of histidine and peptides containing histidyl residues.[6,7] Furthermore, it has been reported that ionization of the pyrrolic-NH group of histidine may also be influenced by mixed-complex formation.[6] As a consequence, the stability constants of various ternary complexes with CH_2R_2, R_2CH-CH_2CO_2H and R_2CHNH_2 and the results obtained for the ionization of N^1H of co-ordinated imidazole are also included in this study.



CH_2R_2



$R^1 = H$ R_2CHNH_2
$R^1 = MeCO\text{-}Pro\text{-}Leu\text{-}Gly$ $MeCO\text{-}Pro\text{-}Leu\text{-}Gly\text{-}NHCHR_2$
$R^1 = Bu^tOCO\text{-}Pro\text{-}His\text{-}Gly$ $Bu^tOCO\text{-}Pro\text{-}His\text{-}Gly\text{-}NHCHR_2$



$R^2 = OH$ $R_2CHCH_2CO_2H$
$R^2 = Ile\text{-}Ala\text{-}Gly\text{-}OEt$ $R_2CHCH_2CO\text{-}Ile\text{-}Ala\text{-}Gly\text{-}OEt$
$R^2 = Ile\text{-}His\text{-}Gly\text{-}OEt$ $R_2CHCH_2CO\text{-}Ile\text{-}His\text{-}Gly\text{-}OEt$

Experimental

Materials.—The procedure for the preparation of bis(imidazolyl) ligands and peptides containing C- or N-terminal bis(imidazol-2-yl) residues has already been reported elsewhere.[8] The purity of the ligands was checked by chromatographic methods and potentiometric titrations. The other chemicals were purchased from Sigma and used without further purification. The metal-ion stock solutions were prepared from analytical grade reagents and their concentration was checked gravimetrically.

Potentiometric Measurements.—The pH-metric titrations were performed on 5 cm^3 samples in the concentration range 2×10^{-3}–8×10^{-3} mol dm^{-3} at metal-ion to ligand ratios between 1:1 and 1:3. Argon was bubbled through the samples to ensure the absence of oxygen and for stirring the solutions. All pH-metric measurements were carried out at 298 K and at a constant ionic strength of 0.2 mol dm^{-3} (KCl). Measurements were made in a Radiometer TTA 80 titration unit with a pHM 84 pM-meter equipped with G2040 B glass and K4040 calomel electrodes and a ABU 13 autoburette containing carbonate-free potassium hydroxide in known concentration. The pH readings were converted to $[H]^+$ ion concentration as described previously.[9,10] Protonation constants of the ligands and the overall stability constants of the binary and ternary systems were calculated using a general computational program (PSEQUAD).[11]

Spectroscopic Measurements.—Visible spectra of the copper(II) complexes were recorded on Beckman ACTA MIV or JASCO UVIDEC-610 double-beam spectrophotometers in the same concentration range as used for the potentiometry. Anisotropic X-band EPR spectra (9.15 GHz) of frozen solutions were recorded at 120 K, using a Varian E-9 spectrometer after addition of ethylene glycol to ensure good glass formation in frozen solutions.

Results and Discussion

Protonation constants of the ligands are included in Table 1. It can be seen that deprotonation of imidazole nitrogens of bis(imidazole) ligands occurs at lower pH values than that of free imidazole, as the interaction of aromatic rings decreases the basicity of nitrogen donors. This effect, however, depends on several factors including the length of carbon chain between the aromatic rings and the substituents present on the linking carbon atom. A survey of literature data on ligands containing directly coupled aromatic nitrogen bases shows that the first

Table 1 Protonation constants of the ligands [T = 298 K; I = 0.2 mol dm^{-3} (KCl)]*

Ligand	pK_1	pK_2	pK_3
CH_2R_2	4.74(1)	6.93(1)	
R_2CHNH_2		4.07(1)	6.49(1)
Pro-Leu-Gly-$NHCHR_2$	3.31(1)	5.67(1)	
Bu^tOCO-Pro-His-Gly-$NHCHR_2$	3.11(1)	5.42(1)	6.38(1)
$R_2CHCH_2CO_2H$	2.79(1)	4.62(1)	6.90(1)
R_2CHCH_2CO-Ile-Ala-Gly-OEt	3.82(1)	5.99(1)	
R_2CHCH_2CO-Ile-His-Gly-OEt	4.01(1)	5.67(1)	6.65(1)
Imidazole	6.95		

* All pK values refer to imidazole nitrogens except pK_3 = 6.49 for R_2CHNH_2 (amino group) and pK_1 = 2.79 for $R_2CHCH_2CO_2H$ (carboxylic group).

pK value is so low that it cannot be determined pH-metrically [as is the case for 2,2′-bis(imidazole)],[12] or is very low [$pK_1 = -0.7$ for 2-(pyridin-2-yl)imidazole or $pK_1 = -0.2$ for 2,2′-bipyridine].[13] This is also supported by the investigations of Bühler and Anderegg,[14] who determined the pK values of a series of bis(pyridin-2-yl)alkanes with an increasing number of carbon atoms between the pyridine rings and found that longer aliphatic chains had less effect on the protonation processes. In the case of imidazole and imidazol-4-yl derivatives (*e.g.* histidine) protonation of N^1 and N^3 nitrogens results in the existence of various tautomers.[15] The derivatives containing imidazol-2-yl groups are, however, completely symmetrical and nitrogen atoms cannot be distinguished.

In the case of the derivatives of bis(imidazol-2-yl)methane (CH_2R_2) pK values are also influenced by the substituents of the methine group. Among the ligands studied in this work the free amino group has the most pronounced effect in this respect (see data for R_2CHNH_2). The positive charge of the protonated ammonium group will result in a further decrease in the basicity of aromatic nitrogens and the first pK value of R_2CHNH_2 cannot be determined potentiometrically ($pK \leqslant 1.5$). If the amino group is converted to an amide nitrogen (see data for peptides with C-terminal $NHCHR_2$) the decrease in pK values is smaller than for R_2CHNH_2, but the imidazole nitrogens are still less basic than those of CH_2R_2.

When the two imidazole rings are attached to propionic acid, as in $R_2CHCH_2CO_2H$, pK values for the imidazole nitrogens are barely affected. However, if the carboxylate is converted to an amide at the N-terminal part of a peptide (see data for R_2CHCH_2CO peptides) a decrease in the basicity of imidazole nitrogens is again observed.

There are additional imidazole nitrogen donors in the side-chain residues of the peptides Bu'OCO-Pro-His-Gly-NHCHR$_2$ and R$_2$CHCH$_2$CO-Ile-His-Gly-OEt, but as they are quite far from the bis(imidazole group) these nitrogens act as independent binding sites and their pK values are very similar to those of tripeptides containing internal histidyl residues (*e.g.* pK = 6.35 for Gly-His-Gly).[16]

Stability constants of the copper(II) complexes of bis-(imidazolyl) ligands are given in Table 2, revealing that the formation of various mono- and bis-(ligand) complexes are characteristic of all bis(imidazolyl) ligands. In case of CH$_2$R$_2$ and the peptides MeCO-Pro-Leu-Gly-NHCHR$_2$ and R$_2$CHCH$_2$CO-Ile-Ala-Gly-OEt the imidazole nitrogens are the exclusive binding sites for metal-ion co-ordination. Thus, the titration curves can be fitted assuming formation of the species [CuA]$^{2+}$ and [CuA$_2$]$^{2+}$, in which there is equatorial co-ordination of two and four imidazole nitrogen atoms, respectively. Formation of these complexes occurs in an acidic pH range 2–5, which corresponds well to the high values obtained for the stability constants. The co-ordination of the second ligand is generally slightly hindered and this is reflected in the high ratio of stepwise stability constants (see log K_1/K_2 values in Table 2). In the case of common peptides the amide nitrogens are generally involved in complex formation with copper(II) ions.[17,18] The formation of the amide-bound peptide complexes, however, requires the presence of an anchoring donor group, which should be in a chelating position with respect to the amide groups (a terminal amino group is the most common anchor). The nitrogens of bis(imidazolyl) residues of peptides can, however, form stable chelate rings with each other. Accordingly, they will prevent co-ordination of amide nitrogens, which does not occur even in basic solutions.

Visible and EPR spectroscopic data support these findings and measurements of the former (together with the binding modes of ligands suggested) are given in Table 3. Assuming equatorial co-ordination of four imidazole nitrogen atoms around copper(II) ions the absorption maxima should occur at λ = 581 nm, according to calculations performed by Billo.[19] The values obtained for CH$_2$R$_2$, MeCO-Pro-Leu-Gly-NHCHR$_2$ and R$_2$CHCH$_2$CO$_2$H are in good agreement with this. Absorption maxima for the bis(ligand) complexes of R$_2$CHCH$_2$CO-Ile-Ala-Gly-OEt cannot be determined because of precipitation.

EPR measurements were carried out on the copper(II) complexes of CH$_2$R$_2$, R$_2$CHNH$_2$, R$_2$CHCH$_2$CO$_2$H and R$_2$CHCH$_2$CO-Ile-His-Gly-OEt and the most important parameters are included in Table 4, while selected spectra are shown in Fig. 1. The data for the [CuA]$^{2+}$ and [CuA$_2$]$^{2+}$ complexes of CH$_2$R$_2$ correspond very well to co-ordination of two and four nitrogen donors, respectively, and the appearance of the well resolved ^{14}N superhyperfine splitting [*ca.* 1.5 mT, see Fig. 1(*b*)] definitely supports this co-ordination mode.

Protonated and/or deprotonated complexes are also formed with the ligands containing additional donor groups (*e.g.* carboxylate for R$_2$CHCH$_2$CO$_2$H, amino for R$_2$CHNH$_2$ and imidazole-*N* of histidyl side chains of Bu'OCO-Pro-His-Gly-NHCHR$_2$ and R$_2$CHCH$_2$CO-Ile-His-Gly-OEt). The visible absorption and EPR parameters for R$_2$CHCH$_2$CO$_2$H are very similar to those of CH$_2$R$_2$, suggesting that imidazole nitrogens are the main binding sites and that they are co-ordinated equatorially. The species [CuAH]$^{2+}$ and [CuA$_2$H]$^{+}$ correspond to complexes with protonated carboxylate groups, while [CuA$_2$H$_2$]$^{2+}$ is not present in measurable concentration due to the relatively low protonation constant of the carboxylate group. Upon deprotonation of these species a weak axial interaction of the carboxylate group cannot be excluded in the species [CuA]$^{+}$ and [CuA$_2$], the existence of which is reflected in the high values obtained for the stability constant of [CuA]$^{+}$ and in the increase of the ratio of stepwise stability constants.

Complex formation processes of the copper(II)–R$_2$CHNH$_2$ system are more complicated than those of CH$_2$R$_2$ or R$_2$CHCH$_2$CO$_2$H. The amino group, which can form a five-membered chelate ring with the copper and imidazole nitrogens, co-ordinates the metal much more effectively than the carboxylate group. Its pK value is, however, significantly higher than those of the imidazole residues, and therefore the species formed in very acidic solutions (the fully protonated species [CuAH]$^{3+}$ and [CuA$_2$H$_2$]$^{4+}$) are the same 4N-co-ordinated complexes as those obtained for CH$_2$R$_2$ or R$_2$CHCH$_2$CO$_2$H {see EPR spectra of [CuA$_2$H$_2$]$^{4+}$ in Fig. 1(*c*)}. It is also notable that formation of the protonated complexes occurs below pH 2, thus the stability constants for [CuAH]$^{3+}$ are relatively uncertain. Deprotonation of the non-co-ordinated ammonium groups is accompanied by characteristic changes in both the absorption and EPR spectra and these changes depend on the metal-ion to ligand ratios. In the presence of excess of ligand (in 1:2 solutions) the absorption maximum appears at λ = 597 nm, which indicates a small red shift upon deprotonation of the

Table 2 Stability constants (log β_{pqr}) of copper(II) complexes [T = 298 K, I = 0.2 mol dm^{-3} (KCl)]

	MA	MA$_2$	MAH	MA$_2$H	MA$_2$H$_2$	M$_2$A$_2$H$_{-1}$	M$_2$A$_2$H$_{-2}$	log(K_1/K_2)
CH$_2$R$_2$	9.64(1)	17.03(1)	—	—	—	—	—	2.25
MeCO-Pro-Leu-Gly-NHCHR$_2$	8.65(3)	15.24(3)	—	—	—	—	—	2.06
R$_2$CHCH$_2$CO-Ile-Ala-Gly-OEt	8.92(3)	15.52(3)	—	—	—	—	—	2.32
R$_2$CHCH$_2$CO$_2$H	10.13(1)	17.29(3)	13.36(2)	21.20(3)	—	—	—	2.97
Bu'OCO-Pro-His-Gly-NHCHR$_2$	9.51(2)	15.69(2)	14.70(2)	21.88(3)	27.44(2)	—	—	3.33
R$_2$CHCH$_2$CO-Ile-His-Gly-OEt	10.05(4)	14.74(10)	14.64(2)	21.08(12)	26.68(6)	—	—	5.35
R$_2$CHNH$_2$	9.89(1)	16.89(2)	14.0(20)	21.50(2)	25.21(1)	18.43(5)	12.78(4)	2.89

Table 3 Spectral parameters and binding modes[a] of various bis(ligand) complexes of copper(II)

			Co-ordination	
Ligand	pH	λ_{max}/nm	Equatorial	Axial
CH$_2$R$_2$	5.1	578	4 N (im)	
R$_2$CHCH$_2$CO$_2$H	7.2	580	4 N (im)	COO$^-$
R$_2$CHNH$_2$	> 5	597	3 N (im) + NH$_2$	N (im)
MeCO-Pro-Leu-Gly-NHCHR$_2$	6.2	583	4 N (im)	
Bu'OCO-Pro-His-Gly-NHCHR$_2$	6.5	598	4 N (im)	N (His)
R$_2$CHCH$_2$CO-Ile-His-Gly-OEt	4.5	620	4 N (im)	CO (peptide)[b]
	6.5	683[c]	4 N (im)	N (His)[d]

[a] Abbreviations for binding modes: im = imidazole N, His = histidyl N. [b] [CuA$_2$H$_2$]$^{4+}$. [c] Overlap of species. [d] [CuA$_2$]$^{2+}$.

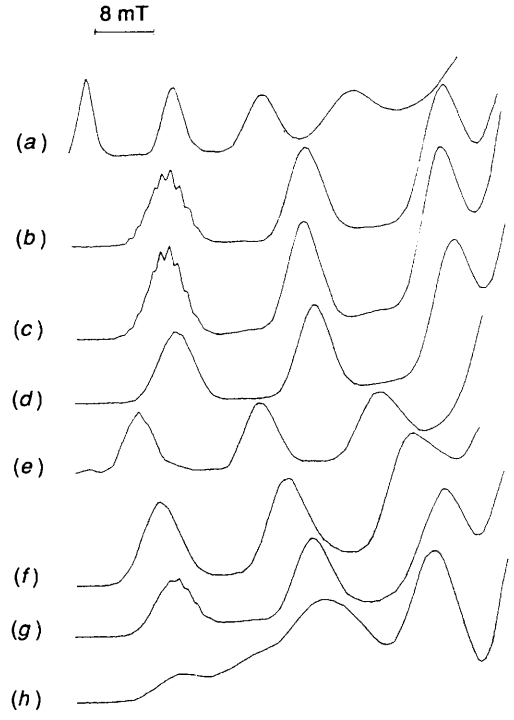


Fig. 1 Frozen solution EPR spectra (parallel region) of copper(II) complexes at pH 7 (major species): (*a*) $[Cu(H_2O)_6]^{2+}$; (*b*) $[CuA_2]^{2+}$ (A = CH_2R_2); (*c*) $[CuA_2H_2]^{4+}$ (A = R_2CHNH_2); (*d*) $[CuA_2]^{2+}$ (A = R_2CHNH_2); (*e*) $[CuAH]^{3+}$ (A = R_2CHCH_2CO-Ile-His-Gly-OEt); (*f*) $[CuA]^{2+}$ (A = R_2CHCH_2CO-Ile-His-Gly-OEt); (*g*) $[CuA_2H_2]^{4+}$ (A = R_2CHCH_2CO-Ile-His-Gly-OEt); (*h*) $[CuA_2]^{2+}$ (A = R_2CHCH_2CO-Ile-His-Gly-OEt)

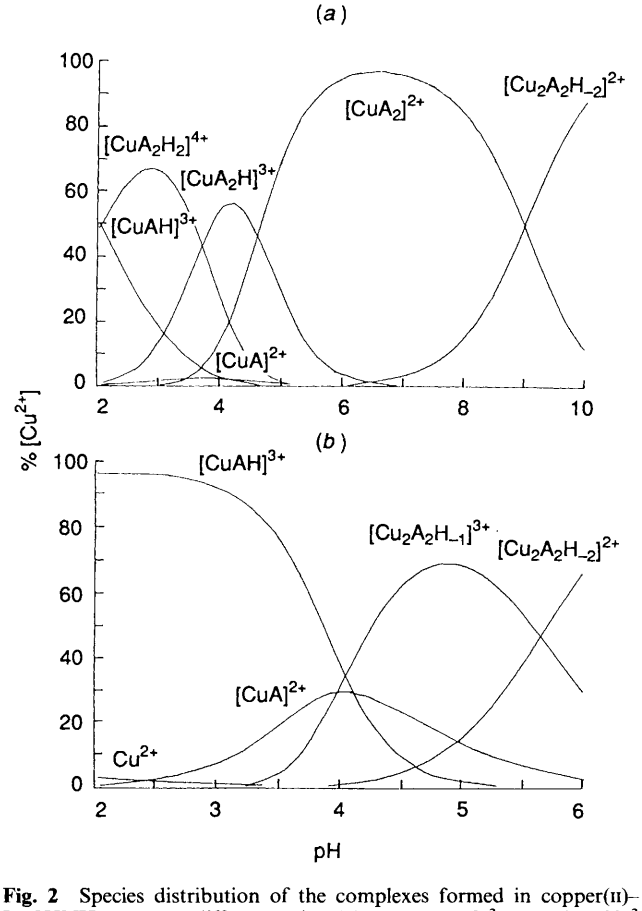


Fig. 2 Species distribution of the complexes formed in copper(II)–R_2CHNH_2 system at different ratios: (*a*) $c_A = 8 \times 10^{-3}$, $c_M = 4 \times 10^{-3}$ mol dm^{-3}; (*b*) $c_A = c_M = 4 \times 10^{-3}$ mol dm^{-3}

Table 4 EPR spectral data of copper(II) complexes with bis(imidazol-2-yl) ligands (T = 120 K)

Ligand	Complex	$g_{\|}$	$A_{\|}/10^{-4}$ cm^{-1}
CH_2R_2	$[CuA]^{2+}$	2.307	181
	$[CuA_2]^{2+}$	2.237	199
$R_2CHCH_2CO_2H$	$[CuAH]^{2+}$	2.298	179
	$[CuA_2]$	2.235	202
R_2CHNH_2	$[CuAH]^{3+}$	2.303	176
	$[CuA_2H_2]^{4+}$	2.239	198
	$[CuA_2H]^{3+}$	2.234	199
	$[CuA_2]^{2+}$	2.227	200
2-Aminomethylpyridine	$[CuA]^{2+}$	2.295	178
	$[CuA_2]^{2+}$	2.220	198
R_2CHCH_2CO-Ile-His-Gly-OEt	$[CuAH]^{3+}$	2.297	180
	$[CuA_2H_2]^{4+}$	2.234	191
	$[CuA]^{2+}$	2.264	183
	$[CuA_2]^{2+}$	2.324	141

ammonium groups of $[CuA_2H_2]^{4+}$ (λ_{max} = 580 nm) suggesting axial co-ordination of at least one nitrogen donor in the species $[CuA_2]^{2+}$. The data obtained in equimolar solutions and the EPR measurements suggest that one of the imidazole nitrogens is co-ordinated axially and it is replaced by the amino group in the equatorial plane. The EPR data change slightly during deprotonation of the bis(ligand) complexes of R_2CHNH_2, but the loss of detectable superhyperfine splitting [see Fig. 1(*d*)] supports the non-equivalence of nitrogen donors around copper(II). It is also supported by the small decrease in $g_{\|}$, which, for $[CuA_2]^{2+}$, is just the average of those of the bis(ligand) complexes of CH_2R_2 (co-ordination of four imidazole N) and 2-aminomethylpyridine (co-ordination of two aromatic and two amino nitrogens). The similar tridentate co-ordination of bis(pyridin-2-yl)methylamine (a structural analogue of R_2CHNH_2) has already been suggested in the solid state.[20]

Tridentate co-ordination of R_2CHNH_2 is more evident in the complex formed in equimolar solution. In this case the deprotonation of the ammonium group is followed by the consumption of one more equivalent of base in the pH-metric titrations. This can only be explained by the formation of hydroxo complexes and titration curves can be fitted with the existence of either monomeric $[CuAH_{-1}]^+$ or dimeric $[Cu_2A_2H_{-2}]^{2+}$ species. The visible absorption and EPR spectra of the hydroxo complexes, however, suggest that dinuclear complexes are favoured. Species distribution of the complexes formed in the copper(II)–R_2CHNH_2 system at different ratios is shown in Fig. 2. Around the physiological pH range the bis(ligand) complex predominates in the presence of excess of ligands, while the dinuclear hydroxo species is present in equimolar systems. Dimeric hydroxo complexes of copper(II) are generally hydroxo-bridged dimers with short copper(II)–copper(II) distances, which results in antiferromagnetically coupled and EPR-silent species.[21] The absorption maximum of $[Cu_2A_2H_{-2}]^{2+}$ is, however, around 595 nm, which suggests equatorial co-ordination of at least three nitrogen atoms. Due to steric reasons all the three nitrogens of R_2CHNH_2 cannot co-ordinate equatorially to the same metal ion, but we suggest that three co-ordination sites of each copper(II) are occupied by an imidazole nitrogen, an amino nitrogen and a hydroxo group, while the second imidazole behaves as a bridging ligand saturating the co-ordination sphere of copper(II) ions. The EPR spectra of equimolar solutions of copper(II) and R_2CHNH_2 are shown in Fig. 3 as a function of pH and they support this assumption; the EPR parameters do not change significantly upon the formation of hydroxo complex, but a poorly resolved and broad EPR signal is obtained. It also can be seen from Fig. 3 that the dimer is definitely an EPR-active species and the line broadening can probably be attributed to the dipolar coupling of the imidazole-bridged copper(II) ions.

In the peptides Bu^{t}OCO-Pro-His-Gly-NHCHR$_2$ and R$_2$-

$CHCH_2CO$-Ile-His-Gly-OEt there is an additional imidazole residue in the molecules. It is, however, too far from the bis(imidazolyl) moiety to form a stable chelate ring. On the other hand, the imidazole nitrogen donor atom of the histidyl residue could be an anchor for copper(II)-induced amide deprotonation and co-ordination of the peptide molecules. However, both potentiometric and spectroscopic measurements undoubtedly prove that amide co-ordination of peptide residues could not compete with the co-ordination of bis(imidazol-2-yl) residues in this case. The data collected in Table 2 reveal that mono- and bis-(ligand) complexes are also formed with these peptides but that the presence of histidyl residues influences the stability of the complexes. For Bu^tOCO-Pro-His-Gly-$NHCHR_2$ the absorption maximum of the species $[CuA_2]^{2+}$ is slightly shifted to higher wavelengths (λ = 598 nm) suggesting axial co-ordination of the side-chain imidazole residue. The tridentate co-ordination of at least one of the ligands is supported by the increased log K_1/K_2 ratio in the bis(ligand) complex. The ratio of the stepwise stability constants is 1.96 for protonated species and is similar to that of CH_2R_2 or other 4N-co-ordinated species. The value of log K_1/K_2 is, however, increased to 3.33 for the species $[CuA_2]^{2+}$, which suggests some change in the co-ordination geometry.

The ratio of the stepwise stability constants is particularly high for the copper(II)–R_2CHCH_2CO-Ile-His-Gly-OEt system, with log(K_1/K_2) 2.60 and 5.35 before and after the deprotonation of the side-chain imidazole group, respectively. This large difference suggests a change of geometry, probably resulting from the co-ordination of the histidyl residue. Visible and EPR spectroscopic measurements support this conclusion and reveal that the co-ordination mode of the side-chain imidazole residue depends on the metal-ion to ligand ratios. The visible spectral measurements in the copper(II)–R_2CHCH_2CO-Ile-His-Gly-OEt system at different pH values are given in Table 5. The absorption maxima in equimolar solutions are shifted progressively to lower wavelengths. Assuming equatorial co-ordination of three imidazole nitrogens in the species $[CuA]^{2+}$ λ_{max} = 628 nm can be calculated,[19] which is close to the value obtained experimentally. The EPR parameters of this species are in between those of the two- and four-imidazole co-ordinated species [see Table 4 and Fig. 1(*e*)–(*h*)] also suggesting equatorial co-ordination of the side-chain imidazole nitrogen donor atom. The existence of this species can be explained by the formation of a 14-membered macrochelate or 'loop'.

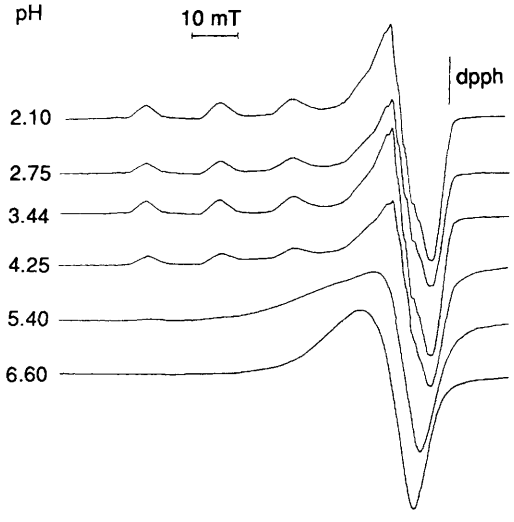


Fig. 3 Frozen solution EPR spectra of equimolar solutions of copper(II) and R_2CHNH_2 as a function of pH (dpph = diphenylpicrylhydrazyl)

For a metal-ion to ligand ratio of 1:2 the absorption maxima are first blue-shifted, which corresponds to the co-ordination of bis(imidazolyl) nitrogen donors. The absorption maximum of $[CuA_2H_2]^{4+}$ (pH = 4.5 in Table 5) appears at λ = 620 nm, which is significantly higher than that of the other species with four co-ordinated imidazole nitrogens. The subsequent red shift of the absorption band suggests axial co-ordination of peptide residues (*e.g.* the carbonyl groups) even in the protonated complexes. The EPR spectrum of the protonated bis(ligand) complex of R_2CHCH_2CO-Ile-His-Gly-OEt, $[CuA_2H_2]^{4+}$, still indicates equatorial co-ordination of four nitrogen donors, but the lowering of $A_{\parallel}$ suggests that, besides axial interaction, there is further distortion in the co-ordination of the metal. Upon deprotonation of the histidyl side chain there is a significant red shift in the absorption spectra, which is accompanied by a change in the EPR spectra [Fig. 1(*g*), (*h*)]. The approximate EPR parameters measured for the species $[CuA_2]^{2+}$, which coexists with $[CuA_2H]^{3+}$ and $[CuA]^{2+}$, suggest decreased tetragonality at copper(II) and equatorial co-ordination of four and axial co-ordination of one (or, more likely, two) additional imidazole nitrogens. The species $[CuA_2H]^{3+}$ does not yield a distinguishable EPR spectrum.

Stability constants obtained for the zinc(II) complexes of bis(imidazolyl) ligands are listed in Table 6. Zinc(II) can also form stable mono- and bis-(ligand) complexes with bis(imidazol-2-yl)methane and its derivatives, in which the imidazole nitrogens are the main binding sites. Stability constants for zinc(II) are lower than those for copper(II), but zinc(II) ions are still bonded around the physiological pH range. Complex formation occurs at higher pH values than for copper(II), and accordingly protonated complexes are only formed with R_2CHNH_2 and the peptides containing histidyl residues in the side chain. The tridentate co-ordination of R_2CHNH_2 in the zinc(II) complexes is supported by the existence of water-soluble hydroxo complexes and a pK value of 7.21 is obtained for the deprotonation of co-ordinated water molecules, which suggests that zinc(II) complexes of bis(imidazolyl) ligands are promising candidates for modelling zinc-containing enzymes. The co-ordination of the imidazole side chains of the histidyl residues is reflected in the increased ratio of stepwise stability constants for the zinc(II) complexes of Bu^tOCO-Pro-His-Gly-$NHCHR_2$ and R_2CHCH_2CO-Ile-His-Gly-OEt. The differences in log K values are, however, much less significant than they were for copper(II) complexes, which can be explained by the preferred octahedral symmetry of zinc(II) complexes.

Table 5 Visible spectral parameters of the copper(II)–R_2CHCH_2CO-Ile-His-Gly-OEt system as a function of pH at different ratios

Cu^{II}:ligand 1:2			Cu^{II}:ligand 1:1		
pH	λ_{max}/nm	ε_{max}/dm^3 mol^{-1} cm^{-1}	pH	λ_{max}/nm	ε_{max}/dm^3 mol^{-1} cm^{-1}
2.71	680	38.0			
3.03	677	40.0	2.91	693	35.3
4.06	630	45.5	3.97	675	38.0
4.52	620	48.0	4.54	650	44.3
5.03	625	50.0	4.97	643	48.7
6.01	652	54.0	5.92	638	54.8
6.55	683	59.0			

Mixed-ligand complex-formation processes were studied for CH_2R_2, $R_2CHCH_2CO_2H$ and R_2CHNH_2 with other ligands (B) including glycine, histidine and tiron (disodium 4,5-dihydroxy-1,3-benzenedisulfonate), which may co-ordinate through NH_2 and CO_2, [NH_2,N(imidazole),CO_2], and O^-,O^-(phenolate), respectively. Stability constants obtained for the mixed-ligand systems are collected in Table 7, which also lists equilibrium data on the binary systems of B ligands taken from the literature.[6] Ternary complexes were formed in all systems studied and their stoichiometry depends on the pK values of the non-co-ordinated functional groups. In case of CH_2R_2 the species $[CuAB]^{2+}$ predominates and log K_M values reveal that the formation of ternary complexes is favoured in all cases. The stabilization of the mixed-ligand complexes follows the trends obtained previously for similar ternary systems. Namely, the higher the difference in the stepwise stability constants of the parent complexes the higher the log K_M values.[22,23] On the other hand, it has been demonstrated for a series of ligands that co-ordination of aromatic nitrogen donors prefers the binding of oxygen (especially phenolate) donors.[23,24] This effect is reflected in the high thermodynamic stability of the mixed-ligand complexes of tiron. For $R_2CHCH_2CO_2H$ and R_2CHNH_2 various protonated species are also present, in which the carboxylate and/or histidine and amino and/or histidine residues are protonated, respectively. The copper(II)–CH_2R_2–tiron system cannot be studied above pH 4.3 due to precipitation.

The formation of the species $CuABH_{-1}$ very probably corresponds to the deprotonation of the pyrrolic N^1H group of the imidazole. The ionization of this group was also followed in the binary copper(II)–CH_2R_2, –$R_2CHCH_2CO_2H$ or –R_2CHNH_2 systems, but deprotonation was not observed below pH 11, and hydrolytic processes hinder pH-metric studies at high pH values. It is clear from Table 7 that ionization of N^1H is most favoured in ternary complexes of $R_2CHCH_2CO_2H$, which can be explained by the different charge of complexes of $R_2CHCH_2CO_2H$. The decrease in pK values, compared to the free ligands, is about four to five orders of magnitude and it is notable that deprotonation also depends on the B ligands. The decrease in pK values is lowest for binary complexes of tiron, which supports the previous observation that phenolate-$O^- \longrightarrow Cu^{2+}$ charge transfer processes decrease the π-bonding character of the Cu^{2+}–N(imidazole) interaction, which results in a relative decrease in the acidity in the $N^1(H)$ of imidazole.

Conclusion

A series of peptides coupled with bis(imidazol-2-yl)methane have been synthesized both to mimic the binding sites of various metalloenzymes and to provide models for selective enzyme inhibition. The combined potentiometric and spectroscopic studies reveal that all the bis(imidazolyl) compounds (CH_2R_2, R_2CHNH_2 and $R_2CHCH_2CO_2H$) and their peptide derivatives (R_2CHCH_2CO-Ile-Ala-Gly-OEt, R_2CHCH_2CO-Ile-His-Gly-OEt, MeCO-Pro-Leu-Gly-$NHCHR_2$ and Bu^{t}OCO-Pro-His-Gly-$NHCHR_2$) form stable mono- and bis-(ligand) complexes with copper- and zinc-(II) ions. The bis(imidazol-2-yl) groups are the main binding sites in these complexes *via* the formation of stable six-membered chelate rings. Tridentate co-ordination can occur with the carboxylate group of $R_2CHCH_2CO_2H$, but it is especially favoured for R_2CHNH_2. In equimolar solutions of copper(II) and R_2CHNH_2 stable dinuclear hydroxo complexes with imidazole nitrogens as bridging ligands are formed, and the $[CuA_2]^{2+}$ complex is concluded to involve axial interaction of at least one imidazole nitrogen donor atom. Zinc(II) ions also form stable mixed-hydroxo complexes with R_2CHNH_2, in which the pK of the co-ordinated water molecules is around the physiological pH range.

From the results obtained on the complexes of the peptides R_2CHCH_2CO-Ile-Ala-Gly-OEt and MeCO-Pro-Leu-Gly-$NHCHR_2$ it can be concluded that peptide residues do not have a significant effect on complex formation with the bis(imidazolyl) ligands, regardless of the location (C- or N-terminal) of the bis(imidazolyl) moiety in the molecules. This is however not the case if strongly co-ordinating donor groups are present in the side chains of peptides. Histidyl residues of Bu^{t}OCO-Pro-His-Gly-$NHCHR_2$ and, especially, R_2CHCH_2CO-Ile-His-Gly-OEt influence the stability and even the binding modes of the ligands. In case of the former ligand there is only a relatively weak axial interaction of the side-chain imidazole residue. The bis(imidazolyl) moiety is in the C-terminal position of the peptide and can form neither a stable chelate nor a loop with the side-chain residue. However, the bis(imidazolyl) group is in an N-terminal position in R_2CHCH_2CO-Ile-His-Gly-OEt, which enables equatorial co-ordination of the side-chain imidazole residue in the species $[CuA]^{2+}$. This species is thought to involve a 14-membered macrochelate or 'loop' around the central copper(II) ions. Similar structures have been published for peptides where the co-ordination of amide nitrogens is hindered and there are additional functional groups in the

Table 6 Stability constants (log β_{pqr}) of zinc(II) complexes [T = 298 K, I = 0.2 mol dm^{-3} (KCl)]

	MA	MA_2	MAH	MA_2H	MA_2H_2	$M_2A_2H_{-1}$	log(K_1/K_2)
CH_2R_2	5.53(1)	10.22(1)	—	—	—	—	0.84
MeCO-Pro-Leu-Gly-$NHCHR_2$	4.85(8)	8.58(4)	—	—	—	—	1.12
R_2CHCH_2CO-Ile-Ala-Gly-OEt	4.92(3)	8.90(5)	—	—	—	—	0.94
Bu^{t}OCO-Pro-His-Gly-$NHCHR_2$	5.96(2)	9.84(2)	10.95(1)	15.83(3)	21.34(2)	—	2.08
$R_2CHCH_2CO_2H$	5.63(1)	10.10(1)	—	—	—	—	1.16
R_2CHCH_2CO-Ile-His-Gly-OEt	5.90(2)	9.70(7)	10.86(2)	15.82(10)	21.23(7)	—	2.1
R_2CHNH_2	5.38(2)	9.92(5)	9.66(4)	14.25(17)	18.85(15)	6.27(6)	0.84

Table 7 Stability constants of mixed-ligand complexes of copper(II) [T = 298 K, I = 0.2 mol dm^{-3} (KCl)]

Ligands (A–B)	log β_{MAB}	log β_{MABH}	log β_{MABH_2}	log $\beta_{MABH_{-1}}$	Δlog K^a	log $K_M{}^b$	δlog (K_1/K_2)	pK_{N^1H}
CH_2R_2–Gly	17.16(2)	—	—	—	0.55	2.45	0.95	—
CH_2R_2–His	18.08(3)	23.39(2)	—	—	1.60	1.31	0.01	—
CH_2R_2–tiron	22.66(2)	—	—	—	0.71	3.21	0.13	—
R_2CHNH_2–Gly	18.25(2)	22.13(6)	—	—	−0.27	4.76	1.62	—
R_2CHNH_2–His	19.00(2)	24.18(2)	28.09(3)	10.17(4)	0.95	3.28	1.62	8.83
R_2CHNH_2–tiron	—	28.56(3)	—	—	—	—	0.54	—
$R_2CHCH_2CO_2H$–Gly	17.77(3)	21.72(8)	—	7.83(6)	0.43	3.41	1.67	9.94
$R_2CHCH_2CO_2H$–His	18.83(2)	24.01(2)	27.49(5)	8.99(4)	1.34	2.55	0.71	9.84
$R_2CHCH_2CO_2H$–tiron	22.81(2)	—	—	12.25(3)	1.05	3.25	0.59	10.56

^{a} $\Delta\log K = \log\beta_{MA} + \log\beta_{MB} - \log\beta_{MAB}$. ^{b} $\log K_M = 2\log\beta_{MAB} - \log\beta_{MA_2} - \log\beta_{MB_2}$.

molecule.[25] This type of co-ordination of the mono(ligand) complex significantly hinders formation of the bis(ligand) complex, which is reflected in the very high ratio of stepwise stability constants. However, the bis(ligand) complex of copper(II) with R_2CHCH_2CO-Ile-His-Gly-OEt dominates in the presence of excess of ligand and around the physiological pH range, where five or six imidazole nitrogens are co-ordinated to the copper(II) ion.

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References

1 C. N. C. Drey and J. S. Fruton, *Biochemistry*, 1965, **4**, 1.
2 C. N. C. Drey and J. S. Fruton, *Biochemistry*, 1965, **4**, 1258.
3 C. C. Tang, D. Davalian, P. Huang and R. Breslow, *J. Am. Chem. Soc.*, 1978, **100**, 3918.
4 M. S. Mohan, *Ind. J. Chem., Sect. A*, 1981, **20**, 252.
5 W. H. Johnson, N. A. Roberts and N. Barkakoti, *J. Enz. Inhib.*, 1987, **2**, 1.
6 I. Sóvágó, T. Kiss and A. Gergely, *J. Chem. Soc., Dalton Trans.*, 1978, 964.
7 I. Sóvágó, E. Farkas and A. Gergely, *J. Chem. Soc., Dalton Trans.*, 1982, 2159.
8 Zs. Likó and H. Süli-Vargha, *Tetrahedron Lett.*, 1993, **34**, 1673.
9 A. Gergely and I. Nagypál, *J. Chem. Soc., Dalton Trans.*, 1977, 1104.
10 H. Irving, G. Miles and L. D. Pettit, *Anal. Chim. Acta*, 1967, **38**, 475.
11 L. Zékány and I. Nagypál, in *Computational Methods for the Determination of Stability Constants*, ed. D. Leggett, Plenum, New York, 1985.
12 F. Holmes, K. M. Jones and E. G. Torrible, *J. Chem. Soc. A*, 1961, 4790.
13 W. J. Eilbeck and F. Holmes, *J. Chem. Soc. A*, 1967, 1777.
14 H. Bühler and G. Anderegg, *Chimia*, 1970, **24**, 433.
15 T. Gajda, B. Henry and J. J. Delpuech, *J. Chem. Soc., Perkin Trans. 2*, 1994, 157.
16 E. Farkas, I. Sóvágó, T. Kiss and A. Gergely, *J. Chem. Soc., Dalton Trans.*, 1984, 611.
17 H. Sigel and R. B. Martin, *Chem. Rev.*, 1982, **82**, 385.
18 I. Sóvágó, in *Biocoordination Chemistry*, ed. K. Burger, Ellis Horwood, New York, 1990.
19 E. J. Billo, *Inorg. Nucl. Chem. Lett.*, 1974, **10**, 613.
20 P. V. Bernhardt, P. Comba, A. Mahu-Rickenbach, S. Stebler, S. Steiner, K. Várnagy and M. Zehnder, *Inorg. Chem.*, 1992, **31**, 4194.
21 A. Gergely and I. Sóvágó, *Inorg. Chim. Acta*, 1976, **20**, 19.
22 I. Sóvágó and A. Gergely, *Inorg. Chim. Acta*, 1976, **20**, 27.
23 I. Sóvágó and A. Gergely, *Inorg. Chim. Acta*, 1979, **37**, 233.
24 H. Sigel, *Angew. Chem., Int. Ed. Engl.*, 1975, **14**, 394.
25 L. D. Pettit, I. Steel, T. Kovalik, H. Kozlowski and M. Bataille, *J. Chem. Soc., Dalton Trans.*, 1985, 1201.

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