A Thermodynamic and Spectroscopic Study of the Proton and Copper(II) Complexes of L-Prolyl-L-histidine, D-Prolyl-L-histidine, L-Histidyl-L-histidine, and D-Histidyl-L-histidine†

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The syntheses of optically pure L-Pro-L-His, D-Pro-L-His, L-His-L-His, and D-His-L-His (HL) (Pro = proline, His = histidine) are reported together with the results of a potentiometric and spectroscopic study of their proton and copper(II) complexes at 25 °C and I = 0.10 mol dm^{-3} (KNO_3). While stereoselectivity is present between both pairs of diastereoisomers, the co-ordination sequences are similar. The Pro-His isomers form the series of complexes [CuL], [$CuH_{-1}L$], [$CuH_{-2}L$], and [$CuH_{-3}L$] (charges omitted), His-His isomers form the series [$Cu(H_2L)$], [Cu(HL)], [CuL], [$Cu_2H_{-1}L_2$], and [$(CuH_{-1}L)_2$] probably returning to the monomer [$CuH_{-2}L$] above pH 10. Both the His-His diastereoisomers form dimers above pH 6.5 with each Cu^{2+} ion co-ordinated to four nitrogen donors. The stereoselectivity in both proton and copper(II) complexes may be explained by considering the preferred *trans* conformations of the peptide chains.

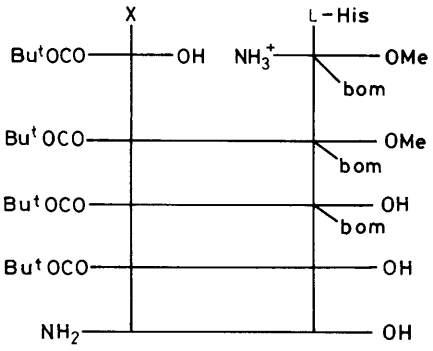
The bis complexes formed by copper(II) with histidine (His) do not show significant stereoselectivity between the species [Cu(L-HisO)$_2$] and [Cu(L-HisO)(D-HisO)] (HisO = histidinate anion), but this has been shown to result from a cancelling of stereoselectivity in the enthalpy and entropy changes accompanying complex formation.[1] When ternary complexes are studied, however, those containing His as one of the ligands often show large stereoselectivity.[2] This is probably a result of the co-ordinating power of the pyridine-like nitrogen atom of the imidazole ring which, amongst all amino acids, makes histidine the residue best able to bond to metal ions through its side-chain when incorporated into a peptide sequence. Hence histidine is the most important amino acid residue in the bioinorganic chemistry of metal–peptide interactions. Proline (Pro) does not contain a co-ordinating side-chain, but is the only naturally occurring amino acid which has a secondary nitrogen atom. Among dipeptides, significant stereoselectivity is usual in the stabilities of [$CuH_{-1}L$] complexes formed by Cu^{2+} with chiral depeptides (*e.g.* L-aa-L-aa where aa signifies an amino acid residue) and *meso* dipeptides (*e.g.* D-aa-L-aa), complexes of the chiral depeptides being the more stable by 0.2—0.8 log units.[3,4] This effect does not depend on the presence of a co-ordinating side-chain and has been shown to result from the minimization of the free energy of the solvent–complex interface by the optimization of non-covalent hydrophobic and electrostatic interactions.[5] Many diastereoisomeric pairs of dipeptides have been studied, but none involving histidine. Peptides containing His which have been studied include Gly-His and His-Gly[6–8] and tripeptides containing these sequences such as Gly-His-Gly,[7] His-Gly-Gly,[8] Gly-Gly-His,[9] and Gly-His-Lys,[10] but in no case has the possibility of stereoselectivity been investigated by studying optically pure dipeptides of His with another chiral amino acid. In particular, complexes of optically pure His-His have not been studied, largely as a result of the ready occurrence of side-chain-induced intramolecular racemization which can occur during peptide synthesis if there is a lone pair of electrons on the π-nitrogen atom. Protonation constants for the optically pure isomers have been calculated from a ^{1}H n.m.r. study of solutions of D/L-His-D/L-His[11] and metal complexes of D/L-His-D/L-His have been studied potentiometrically,[12] but since so many isomers are possible it is difficult to interpret the results. Synthesis of optically pure peptides containing His has been facilitated by the introduction of a new synthetic route which preserves the asymmetry of the chiral centre,[13] and this has allowed the synthesis of chiral dipeptides which are of interest to the co-ordination chemist.

We now report the synthesis of two diastereoisomeric pairs of dipeptides, and the results of a potentiometric and spectroscopic study of their copper(II) complexes. The pairs of dipeptides synthesized were Pro-His and D-Pro-His, and His-His and D-His-His (following the usual convention, the chirality of optically pure L-amino acids is omitted). Proline was selected as a chiral N-terminal amino acid because, while only the N_α atom can act as a donor centre, it can profoundly affect the conformation of a peptide chain. The optical purity of the dipeptides was confirmed by high-field n.m.r. spectroscopy. Histidyl dipeptides contain three different nitrogen donor atoms, the amide nitrogen of the peptide bond, an imidazole nitrogen [im(N_π)], and an N-terminal nitrogen (N_α). These nitrogens can form bonds to Cu^{II} of comparable strength, but chelation of all three donor centres of a particular His sub-unit to the same copper ion is sterically impossible when His is the N-terminal residue and under these conditions the formation of binuclear complexes is encouraged.[12] As a result a wide range of complexed species is to be expected and it is possible that these could show significant stereoselectivity in their stabilities. Hence the interest in the diastereoisomeric pair His-His and D-His-His.

Experimental

Dipeptide syntheses were performed according to the procedure of Brown *et al.*,[13] using the benzyloxymethyl group (bom) for

† *Non-S.I. unit employed:* G = 10^{-4} T.



Scheme. X = L-His, D-His, L-Pro, and D-Pro

the protection of the imidazole side-chains, according to the Scheme. Dipeptides were purifed by gel filtration (Sephadex G-15, water as eluant) and lyophilized.

Structural analysis using n.m.r. spectroscopy confirmed the optical purity. With the DL and LL isomers of Pro-His and a field of 500 MHz, the most deshielded aromatic protons were observed as singlets at δ 7.6 p.p.m. When the isomers were mixed both specific singlets could be identified while in the individual dipeptides the peak for the other isomer could not be detected. Moreover the signals observed for the β and γ proline protons of each isomer are very different. Generally these protons give similar signals[14] (as observed with L-Pro-L-His). With D-Pro-L-His the proline proton resonances are moved upfield. This is probably a result of the stacking of the Pro and His rings with the shielding effect of the His ring producing the upfield shift of the β and γ proline protons. With the isomers of His-His the downfield region characteristic of the aromatic protons showed specific resonances at δ 7 p.p.m. in agreement with the results reported by Tanokura.[11] Comparison of the spectra (at 60 MHz) of the LL and DL isomers with that obtained by mixing the isomers confirmed the absence of racemization during peptide coupling.

Potentiometric Studies.—Stability constants for proton and copper(II) complexes were calculated from titrations carried out at 25 °C using total volumes of 1.5 cm^3. Alkali was added from a 0.1- or 0.25-cm^3 micrometer syringe which had been calibrated both by weight titration and the titration of standardized materials. Changes in pH were followed using a glass electrode calibrated in hydrogen-ion concentration with $HClO_4$.[15] All solutions were of ionic strength 0.10 mol dm^{-3} (KNO_3) and peptide concentrations of 0.003 mol dm^{-3}. Calculations were made with the aid of the SUPERQUAD computer program.[16] This allows for the refinement of total ligand concentrations and was able to confirm the purity of the peptides studied and in particular the absence of acetate, a frequent impurity in peptide samples. The dipeptides were found to be free of any impurity able to co-ordinate to H^+ or Cu^{2+}. In all cases, duplicate or triplicate titrations were carried out at Cu:L ratios of 1:1 and 1:2.

Spectroscopic Studies.—Solutions of the same concentrations as those used in the potentiometric studies were employed. Absorption spectra were recorded on a Beckman UV5240 spectrophotometer and circular dichroism (c.d.) spectra on a JASCO-J-20 automatic recording spectropolarimeter. All c.d. spectra are expressed in terms of $\Delta\varepsilon(\varepsilon_l - \varepsilon_r)$. Electron spin resonance (e.s.r.) spectra were obtained on a JEOL JES-ME-3X spectrometer at liquid-nitrogen temperatures and at 9.13 GHz, and on a Brucker ER 420 *X*-band spectrometer at liquid-helium temperatures (6—8 K). Nuclear magnetic resonance (^{1}H n.m.r.) spectra were recorded on a 500-MHz Brucker spectrometer at 300 ± 2 K in D_2O, using solutions of 1 mg cm^{-3}.

Table 1. Logarithmic proton complex stability constants at 25 °C and I = 0.10 mol dm^{-3} (KNO_3), with estimated standard deviations (e.s.d.s) in parentheses

Peptide	log K_{HL}	log β_{H_2L}	log β_{H_3L}	log β_{H_4L}
L-His-L-His	7.79(1)	14.64(1)	20.32(1)	22.93(2)
D-His-L-His	8.06(1)	14.92(1)	20.16(1)	23.07(2)
L-Pro-L-His	8.82(2)	15.66(2)	18.68(3)	
D-Pro-L-His	9.16(1)	15.93(2)	18.84(3)	

Stepwise constants

	log K_{HL}	log K_{H_2L}	log K_{H_3L}	log K_{H_4L}
L-His-L-His	7.79	6.85	5.68	2.61
D-His-L-His	8.06	6.86	5.24	2.91
L-His-L-His[a]	7.71	6.64	5.61	2.39
D-His-L-His[a]	7.80	6.63	5.13	2.58
D/L-His-D/L-His[b]	7.52	6.55	5.15	2.23
L-Pro-L-His	8.82	6.84	3.02	
D-Pro-L-His	9.16	6.77	2.91	
His-Gly[c]	7.70	5.94	2.82	
Gly-His[c]	8.2	6.75	2.46	
Gly-Pro[d]	8.55	2.79		
Pro-Gly[d]	8.98	3.15		

[a] At 37 °C and unknown ionic strength. Values calculated from ^{1}H n.m.r. data, ref. 11. [b] At 37 °C and I = 0.10 mol dm^{-3} (KNO_3), ref. 12. [c] Ref. 6. [d] H. Sigel, *Inorg. Chem.*, 1975, **14**, 1535.

Results and Discussion

Logarithmic protonation constants for the ligands studied are given in Table 1, together with those for related ligands and for protonation of D-His-His and His-His calculated from a ^{1}H n.m.r. study of D/L-His-D/L-His.[11] The constants for both pairs of ligands studied show three distinct protonation steps: protonation of teminal amino (or imino) nitrogens (pH 8—9), the imidazole im(N_π) nitrogens (pH 5—7), and the carboxylate oxygens (pH 2—3). With Pro-His the logarithmic protonation constants for the imino-nitrogens are higher than for the amino-nitrogens of His-His, in line with differences found for the corresponding amino acids where the value for Pro is 8.8 while that for His to 9.13.[1] The value for D-Pro-His is 0.34 log units greater than for Pro-His, while that for D-His-His is 0.27 log units greater than that for His-His. These values are typical of those found for comparable chiral dipeptides[3-5] and result from stabilization of the zwitterion when the NH^+ and CO_2^- groups are close together in the preferred β conformation of the dipeptide as shown in Figure 1. In the LL (or DD) dipeptides these charged centres are considerably further apart with the result that the zwitterion has less favourable stabilization.[5] Stereoselectivity in the protonation constants of the carboxylate groups of dipeptides containing the His residue would not be expected to resemble closely that found among simpler dipeptides since, at low pH, the presence of positively charged imidazole groups would have a large influence. The two im(N_π) protonation constants are clearly macro-constants containing contributions from the protonation of both the N(C) (C-terminal) and N(N) (N-terminal) imidazole nitrogens. Micro-constants for these protonations have been calculated from ^{1}H n.m.r. spectroscopy and they show that the N(C) nitrogen has a higher affinity for protons by about 0.77 log units.[11] Hence the major contribution to K_{H_2L} would be N(C) protonation. The stereoselectivity in the protonation constants is remarkably close to that calculated from n.m.r. spectroscopy. This is very satisfying for two such completely different techniques since, although the absolute values would be expected to differ as a result of temperature differences (12 °C) and the absence of details of the standardization of pH in the n.m.r. work, the relative differences should be the same. There is no stereo-

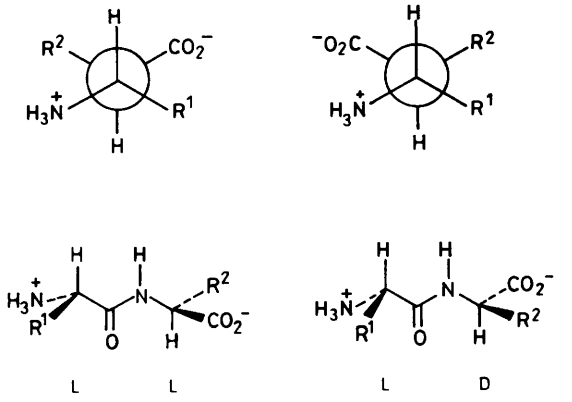


Figure 1. Conformations of chiral dipeptides. The preferred β conformation is the DL isomer

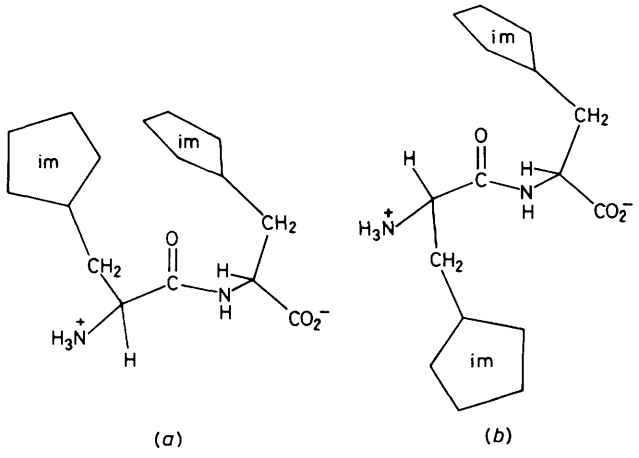


Figure 2. Conformations of (*a*) D-His-His and (*b*) His-His according to ref. 11

selectivity in K_{H_2L} while there is a difference of 0.44 log units in log K_{H_3L}, favouring protonation of the chiral dipeptide, almost identical to that found by Tanokura (0.48 log units).[11] The n.m.r. studies suggest that the dipeptide chains are helical and *trans* about the peptide bond as shown in Figure 2. In this conformation the imidazole rings lie on the same side of the molecule in the *meso* dipeptide and on opposite sides in the chiral molecule. As a result the electrostatic interaction between the imidazole rings of the *meso* molecule would be much greater, causing the considerably larger difference between K_{H_2L} and K_{H_3L} actually found. The stereoselectivity in carboxylate protonation is also comparable to that found in the n.m.r. study, although these constants are more difficult to measure precisely because they are so small. In this protonation reaction, the stereoselectivity is of opposite sign to that found with other dipeptides.[3-5] The reason is immediately apparent from the proposed preferred conformations of the diastereoisomers. In the LL dipeptide the carboxylate group is sterically close to the positively charged protonated C-imidazole ring. This will therefore discourage protonation of the carboxylate oxygen, so lowering the magnitude of the constant. In the preferred conformation of the DL dipeptide the carboxylate and imidazole groups are well separated, resulting in less repulsion to an incoming proton.

Copper complex-formation constants are given in Table 2, together with those for comparable ligands. Spectroscopic properties (visible, c.d., and e.s.r.) are given in Table 3. Species distribution curves for the His-His dipeptides with Cu^{II} are shown in Figure 3.

Table 2. Logarithmic copper(II) complex stability constants at 25 °C and I = 0.10 mol dm^{-3} (KNO_3), with e.s.d.s in parentheses

	log β values				
Peptide	$[Cu(H_2L)]$	$[Cu(HL)]$	$[CuL]$	$[Cu_2H_{-1}L_2]$	$[Cu_2H_{-2}L_2]$
L-His-L-His	19.22(5)	15.77(1)	11.10(2)	19.15(7)	12.70(5)
D-His-L-His	19.56(3)	16.20(3)	12.00(10)	20.2(2)	14.08(12)
D/L-His-D/L-His[a]	17.44	15.19	10.82	18.33	12.39

	$[CuL]$	$[CuH_{-1}L]$	$[CuH_{-2}L]$	$[CuH_{-3}L]$
L-Pro-L-His	10.05(1)	5.43(1)	−4.17(1)	−15.02(2)
D-Pro-L-His	9.89(2)	5.17(1)	−4.67(2)	−16.11(5)
His-Gly[b]	8.83	0.76	−8.74	
Gly-His[b]	9.14	4.89	−4.84	
Gly-Pro[c,d]	6.50			
Pro-Gly[c]	6.42	2.6		

Stepwise protonation constants of complexes

Peptide	log K_1'	log K_2'	log K_3'	log K_4'
L-His-L-His	4.67			
D-His-L-His	4.20			
D/L-His-D/L-His[b]	4.37			
L-Pro-L-His		4.62	9.60	10.85
D-Pro-L-His		4.72	9.84	11.44
His-Gly[b]		8.07	9.50	
Gly-His[b]		4.32	9.73	

$K_1' = [Cu(HL)]/[CuL][H]$, $K_2' = [CuL]/[CuH_{-1}L][H]$, $K_3' = [CuH_{-1}L]/[CuH_{-2}L][H]$, $K_4' = [CuH_{-2}L]/[CuH_{-3}L][H]$

[a] At 37 °C and I = 0.10 mol dm^{-3} (KNO_3), ref. 12. [b] Ref. 6. [c] H. Sigel, *Inorg. Chem.*, 1975, **14**, 1535. [d] log β_{CuL_2} = 11.63.

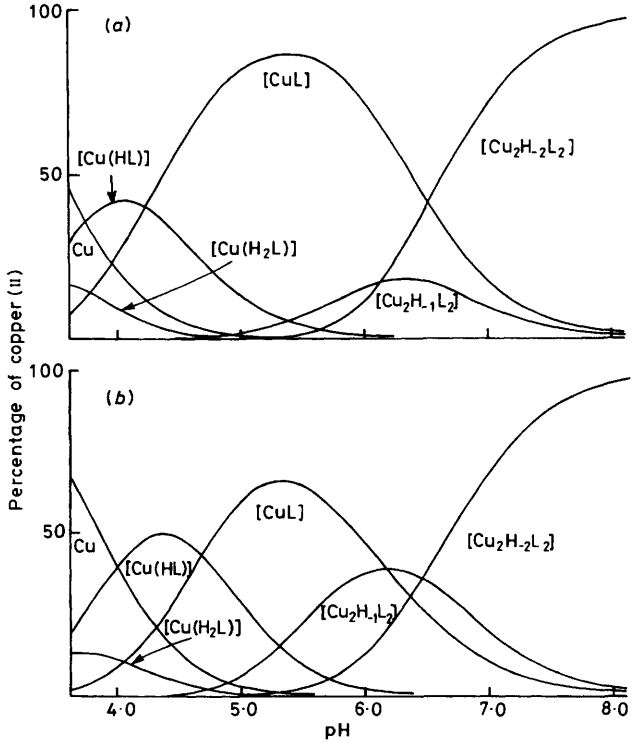


Figure 3. Species distribution curves for (*a*) D-His-His and (*b*) His-His with Cu^{2+} (Cu:L = 1:1, 0.001 mol dm^{-3})

Table 3. Spectroscopic data for copper(II) complexes, Cu:peptide = 1:1, 0.001 mol dm^{-3}

Complex	λ/nm: *d–d* Absorbance maximum	λ/nm: c.d.[a]	Assignment[b]	E.s.r.: $g_{\|}$	E.s.r.: $A_{\|}$/G
(*a*) L-Pro-L-His					
$[CuH_{-1}L]$	580	650 (−0.02)	*B*	2.223	180
		525 (−0.19)	*E*		
		328 (+0.02)	N^-–Cu c.t.		
		268 (+0.2)	NH(Pro)–Cu + π_1N–Cu c.t.[c]		
		237 (−0.7)	Intraligand		
		216 (−1.7)			
$[CuH_{-3}L]$	555	582 (+0.13)	*B*		
		485 (−0.19)	*E*		
		320 (+0.25)	N^-–Cu c.t.		
		258 (+1.3)	NH(Pro)–Cu + π_1N–Cu c.t.		
		227 (+2.4)	Intraligand		
		208 (−2.1)			
(*b*) D-Pro-L-His					
$[CuH_{-1}L]$	580	595 (+0.14)	*B* + *E*	2.223	180[d]
		323 (+0.20)	N^-–Cu c.t.		
		280 (+0.4)	NH(Pro)–Cu + π_1N–Cu c.t.		
		248 (+1.1)	Intraligand		
$[CuH_{-3}L]$	550	480 (−0.05)	*E*		
		315 (sh) (+0.2)	N^-–Cu c.t.		
		280 (br)			
		236 (+2.1)	Intraligand		
(*c*) L-His-L-His					
[Cu(HL)]	650	300 (−)	NH_2–Cu c.t.	2.256	163
		256 (+)	π_1N–Cu c.t.		
[CuL]	615	602 (+0.07)	*B*	2.205	175
		500 (−0.02)	*E*		
		293 (−0.53)	Strong multicomponent band, c.t.		
$[Cu_2H_{-2}L_2]$	550	610 (+0.16)	*B*	2.171	200
		495 (−0.08)	*E*		
		340 (−0.32)	π_2N–Cu c.t.[c]		
		280 (−0.61)	NH_2–Cu c.t.		
		247 (−0.80)	π_1N–Cu c.t.		
		208 (−5.2)	Intraligand		
$[CuH_{-2}L]$	560	635 (+0.17)	*B*		
		495 (−0.10)	*E*		
		267 (+1.1)	Overlapping c.t. bands		
(*d*) D-His-L-His					
[Cu(HL)]		340 (+)	π_2N–Cu c.t.		
		280 (−)	NH_2–Cu c.t.		
[CuL]	610	585 (+0.12)	*B* + *E*	2.223	180
		347 (+0.12)	π_2N–Cu c.t.		
		303 (−0.12)	N^-–Cu c.t.		
		260 (+0.08)	NH_2–Cu + π_1N–Cu c.t.		
		242 (+1.0)	Intraligand		
		215 (−9.5)			
$[Cu_2H_{-2}L_2]$	585	555 (+0.10)	*B*		
		474 (−0.04)	*E*		
		363 (+0.10)	π_2N–Cu c.t.		
		295 (−0.56)	N^-–Cu c.t.		
		257 (+0.71)	NH_2–Cu + π_1N–Cu c.t.		
		221 (−5.5)	Intraligand		
$[CuH_{-2}L]$	560	654 (−0.30)	*B*		
		550 (+0.30)	*E*(1)		
		465 (−0.05)	*E*(2)		
		360 (+0.10)	π_2N–Cu c.t.		
		291 (−0.75)	N^-–Cu c.t.		
		250 (+0.8)	NH_2–Cu + π_1N–Cu c.t.		
		221 (−10)	Intraligand		

[a] $\Delta\varepsilon$/dm^3 mol^{-1} cm^{-1} in parentheses. [b] c.t. = Charge transfer. [c] π_1N– and π_2N–Cu are c.t. transitions from an imidazole nitrogen to Cu^{II}; H. J. Schugar, 'Copper Co-ordination Chemistry: Biochemical and Inorganic Perspectives,' eds. K. D. Karlin and J. Zubieta, Adanine Press, New York, 1983, p. 43. [d] Seven lines of hyperfine structure, resulting from three bound nitrogens.

Both of the Pro-His (HL) diastereoisomers form the series of complexes [CuL], [$CuH_{-1}L$], [$CuH_{-2}L$], and [$CuH_{-3}L$] (charges omitted). Starting at low pH, the first complex to form would be expected to be [Cu(HL)], bonded through either the imino-N [$N(Pro)_\alpha$] and the neighbouring carbonyl oxygen of the peptide linkage (as in Gly-Gly) or through the N_π imidazole nitrogen and the terminal carboxylate oxygen. In both cases the nitrogen atom not co-ordinated would be protonated in this pH region (around pH 4). These complexes could not be detected either potentiometrically or spectroscopically, the first detectable species being [CuL] (pH 4—6). These would be expected to be co-ordinated through both the $N(Pro)_\alpha$ and $im(N_\pi)$ nitrogen-donor centres (NN complexes) but they could not be identified spectroscopically since they have only a small pH range of existence and are overshadowed by the spectrum of the [$CuH_{-1}L$] complexes. However the logarithmic stability constants (around 10) are entirely compatible with such NN co-ordination. The stereoselectivity in formation of the [CuL] complexes is small ($\Delta\log\beta = 0.16$), favouring the chiral complex. This is to be expected if it is assumed that the dipeptide adopts a *trans* conformation around the peptide bond since then the imidazole ring is closer to the N_α donor atom and metal-ion co-ordination is not obstructed by the prolyl ring.

The most important complexes are [$CuH_{-1}L$]. Species-distribution graphs show these to be the major species over the range pH 5—9 and spectroscopic studies (c.d. and e.s.r.) show them unambiguously to be NNN complexes with $N(Pro)_\alpha$, N^-(amido), and $im(N_\pi)$ co-ordination (see Table 3). The stability constants provide evidence in support of this mode of co-ordination since the deprotonation constants of the [CuL] complexes (4.7 log units) are comparable to those for simple dipeptides. The stereoselectivity is again comparatively small ($\Delta\log\beta = 0.26$) and in favour of the chiral complex, as would be expected since there is less interference from the prolyl ring. Around pH 9 [$CuH_{-2}L$] complexes are formed. The deprotonation constants for this reaction ([$CuH_{-1}L$] ⟶ [$CuH_{-2}L$] + H, $\Delta\log\beta$ = about 10 log units) are entirely consistent with the deprotonation of co-ordinated water from an NNN complex. Above pH 10, [$CuH_{-3}L$] species can be detected with deprotonation constants of about 11 log units. It is thought that this represents deprotonation of the pyrrole-like imidazole nitrogen, $im(N_\tau)$, without any change in metal-ion co-ordination. In unco-ordinated peptides this deprotonation does not normally take place until about pH 14,[17] but its basicity is reduced significantly by co-ordination of the $im(N_\pi)$ nitrogen of the ring to Cu^{2+}.[18] The spectroscopic evidence shows all these complexes to be NNN species, but the variation in the signs of the Cotton effect when compared to that for [$CuH_{-1}L$] indicates a major variation in ligand conformation and changes in the absorption maximum from 590 to 560 nm between pH 7 and 11 are compatible with changes in the fourth co-ordination site from H_2O to OH^-. Since the last two deprotonation reactions overlap significantly the constants must be regarded as macro-constants with contributions from both water deprotonation (greatest contribution in the first deprotonation) and $im(N_\pi)$ deprotonation (mostly the second deprotonation). Since there is no change in co-ordination during these deprotonation reactions, the stereoselectivity would be expected to be comparable to that found between the [$CuH_{-1}L$] species. This is, in fact, observed.

As expected, the co-ordination scheme for the His-His diastereoisomers (HL) is more complicated than for Pro-His. The c.d. spectra of the chiral isomer were particularly difficult to resolve as a result of the close proximity of two asymmetric centres and the complicated species equilibria causing many overlapping bands. The first species to be detected unambiguously by both potentiometry and spectroscopy were [CuL]. However from potentiometry the minor species [$Cu(H_2L)$] and [Cu(HL)] preceded the formation of [CuL] in the case of both isomers. These species were also found in the earlier study of D/L-His-D/L-His[12] but since these measurements were made at 37 °C the reported stability constants were lower than those found in this work. The [$Cu(H_2L)$] complexes were very minor species and would be bonded through either the N_α or one of the imidazole $im(N_\pi)$ donors. The [Cu(HL)] complexes exist around pH 4 and, in the case of both isomers, could be identified spectroscopically. The absorption spectrum showed a small plateau around pH 4 with a *d–d* band at 650 nm (typical for an NN species) and, in the c.d. spectrum, bands characteristic of NH_2–Cu and N_π–Cu charge-transfer transitions were present (see Table 3). The presence of an NN complex was confirmed by the e.s.r. spectrum ($g_{\|} = 2.256$ G, $A_{\|} = 163$). The co-ordination centres in this complex would most likely be the terminal N_α and the $im(N_\pi)$ donor of the N-terminal imidazole ring as suggested by the c.d. spectra, forming a six-membered chelate ring. At this pH the $im(N_\pi)$ donor of the other imidazole ring would be protonated. The measured stability constants provide evidence in support of these structures. There is small stereoselectivity in favour of the *meso* complex ($\Delta\log\beta = 0.43$) but, in spite of this, the [Cu(HL)] species is more important with the chiral ligand than with the *meso* isomer because there is even greater stereoselectivity between the [CuL] complexes (see Figure 3).

The [CuL] complexes exist over the range pH 4.5—6 and the spectroscopic studies show them to be NNN complexes. With the *meso* isomer c.d. transitions characteristic of bonding between Cu and N^-, NH_2, and $im(N_\pi)$ were apparent but they were somewhat obscured by strong overlapping with the chiral isomer. However, the absorption maxima (610—615 nm) and the e.s.r. spectra were typical of NNN co-ordination. Although His-His contains four potential N-donor atoms, NNN-bonded complexes can be formed in only one way: using the terminal N_α, the deprotonated peptide N^-, and the $im(N_\pi)$ of the C-terminal imidazole ring, since it is sterically impossible to co-ordinate the N-terminal imidazole nitrogen. The empirical formula [CuL] implies that the $im(N_\pi)$ nitrogen must be protonated, since the neighbouring peptide N has been deprotonated. It is probable that the close proximity of the deprotonated peptide N^- stabilizes the protonated $im(N_\pi)H^+$ groups increasing their pH range of existence towards pH 6, since in the free ligands the logarithmic protonation constants are 5.7 and 5.2 and metal complexation generally decreases ligand protonation constants. From Figure 3 it is seen that the *meso*-[CuL] complex, with a maximum concentration at pH 4.9, is more easily deprotonated than the chiral isomer (maximum at pH 5.3), as would be expected from the stereoselectivity in protonation constants of the ligands. The stereoselectivity in the deprotonation reaction [Cu(HL)] ⟶ [CuL] + H favours the *meso* isomer by 0.5 log units.

Once the second $im(N_\pi)$ donors are deprotonated, co-ordination to Cu^{2+} of either isomer can take place only by dimerization and since this nitrogen donor has a high affinity for Cu^{2+} such dimerization is to be expected. In fact the deprotonated monomer, [$CuH_{-1}L$], could not be detected. The dimer expected would contain two NNNN-bonded copper ions and have the formula [$(CuH_{-1}L)_2$] as shown in Figure 4. Assuming the $im(N_\pi)$ donors bond to copper immediately they are deprotonated, an intermediate dimer of formula [$Cu_2H_{-1}L_2$] is to be expected. Such species were detected potentiometrically, but never as major components, as shown in Figure 3. The major species above pH 6.5 with both isomers were the dimers [$(CuH_{-1}L)_2$]. Their existence was confirmed by potentiometry, carrying out titrations at different concentrations, and by spectroscopy. According to the absorption spectra they are clearly NNNN species (absorption maxima 550—580 nm), while the c.d. spectra confirmed the participation of all donor

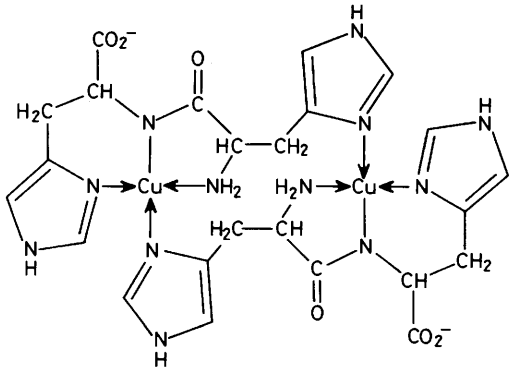


Figure 4. The dimer $[(CuH_{-1}L)_2]$ for His-His

nitrogen atoms in co-ordination. This was confirmed by e.s.r. measurements at liquid-helium temperatures; the spectra were unresolved in liquid nitrogen. While both isomers form dimeric complexes, the stereoselectivity in the stability constants favours that formed by the *meso* isomer, although the species distribution curves are very similar, making stereoselectivity in dimer formation of little practical importance. This is a result of the large stereoselectivity in favour of the *meso* isomer of the [CuL] species which can be attributed to the preferred *trans* conformation of the peptide chain.

While titration data could be taken above pH 10 it proved impossible to fit them satisfactorily by any reasonable model. However, the c.d. spectra suggested significant changes in the conformation and bonding centres at high pH. For example, with the *meso* isomer the decrease in energy of the *B* transition between pH 7 and 10.5 from 603 to 657 nm suggests a change from NNNN to NNN co-ordination. In addition the e.s.r. spectra change above pH 10, with significant narrowing of the lines. These results suggest that, at high pH, the dimers revert to monomers with the probable replacement of the fourth co-ordinated nitrogen by OH^- (hydrolysed water) to give the NNN complexes $[CuH_{-2}L]$, better represented as $[CuH_{-1}L(OH)]$.

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