Studies on Transition-metal–Peptide Complexes. Part 9.† Copper(II) Complexes of Tripeptides containing Histidine

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Copper(II) complexes of the histidine-containing tripeptides glyclyl-L-histidylglycine (GlyHisGly), glycylglycyl-L-histidine (GlyGlyHis), and L-pyroglutamyl-L-histidyl-L-prolinamide (trf), and the dipeptide L-pyroglutamyl-L-histidine methyl ester (pghme), have been studied by pH-metric and spectrophotometric methods. It was found that, similarly as for glycyl-L-histidine (GlyHis), GlyHisGly forms a complex $[CuAH_{-1}]$, but bis complexes are also formed in low concentration. For GlyGlyHis, only the highly stable species $[CuAH_{-2}]^-$ is formed. A complex $[CuAH_{-2}]^-$ is also formed with trf and pghme which indicates that the prolinamide side-chain in trf does not take part in the co-ordination. The sequence of deprotonation of the N^1H group of the imidazole side-chain is as follows: GlyHisGly > GlyHis > pghme > trf > GlyGlyHis.

The main results from the past decade on the complex-forming properties of peptides (HA) were recently reviewed by Sigel and Martin.[1] Their survey clearly shows that research interest is currently centred on identification of the deprotonation processes and the species thereby formed. There is still a continuing interest in the complexes of the histidine-containing peptides, all the more so since the researches have often led to differing results, particularly in the cases of glycyl-L-histidylglycine (GlyHisGly) and glycylglycyl-L-histidine (GlyGlyHis). In an *X*-ray structural study, Österberg *et al.*[2] found that, similarly to glycyl-L-histidine (GlyHis), GlyHisGly co-ordinates to the copper(II) ion *via* the amino-group, the deprotonated peptide nitrogen, and the N^3 atom of imidazole. The resulting monomeric units $[CuAH_{-1}]$ are linked together in the solid state by the chain-terminal carboxyl groups. Recent experiments indicate that GlyHisGly co-ordinates in a similar way in solution,[3] namely deprotonation of the peptide amide group, followed by the formation of polynuclear complexes involving carboxylate bridges. Agarwal and Perrin [4] consider that, with bonding similar to that in the case of GlyHis, both 1 : 1 monomeric and bis complexes of GlyHisGly are formed. This is supported by the e.s.r. results of Sportelli *et al.*[5] and the potentiometric and spectral measurements of Aiba *et al.*[6] The latter authors [6] also observed a further base-consuming process at pH > 9; as concluded earlier [7] for copper(II)–GlyHis, this can presumably be attributed to deprotonation of the N^1H group of imidazole.

Concerning the parent and mixed-ligand complexes of GlyGlyHis with copper(II), some authors [6,8,9] consider that the ligand predominantly forms a complex $[CuAH_{-2}]^-$ with the copper(II) ion, in which co-ordination takes place *via* the amino-group, two deprotonated peptide amide groups, and N^3 of imidazole. Agarwal and Perrin,[10] however, also assume the bis complexes $[CuA_2H_{-1}]^-$ and $[CuA_2H_{-2}]^{2-}$, in which the GlyGlyHis is linked to the metal ion only through N^3 of the imidazole and the neighbouring deprotonated peptide nitrogen. The critical studies by Lau and Sarkar [11] suggest that, besides $[CuAH_{-2}]^-$, in a wide pH range $[CuA_2]$, $[CuA_2H_{-1}]^-$, and $[CuA_2H_{-2}]^{2-}$ are also formed in appreciable concentrations.

Kruck and Sarkar [12] stated that in the copper(II)–GlyGlyHis–histidine system the species $[CuABH_{-1}]^-$ and $[CuABH_{-2}]^{2-}$ (HB = histidine) are formed in considerable concentrations, the histidine displacing the GlyGlyHis imidazole side-chain from the co-ordination sphere. At the same time, Sakurai and Nakahara [8] found that mixed-ligand complex formation can be neglected in the range of formation of $[CuAH_{-2}]^-$; this view is also held by Agarwal and Perrin.[10]

Thyrotropin-releasing factor (trf; L-pyroglutamyl-L-histidyl-L-prolinamide) is one of the most important histidine-containing peptides. Its interaction with copper(II) has been investigated by Kozlowska *et al.*[13,14] From their earlier spectral measurements they concluded that three nitrogen donor atoms and one hydroxide group are co-ordinated to the copper(II), and in addition the N^1H of imidazole undergoes deprotonation at around pH 11—12. According to recent equilibrium and spectral studies [14] the authors point to $[CuA]^+$ and $[CuAH_{-2}]^-$ as the characteristic species. The copper(II) complexes of L-pyroglutamyl-L-histidine, a dipeptide analogue of trf, were interpreted in a similar way as for trf. This would mean that the prolinamide side-chain does not exert an appreciable effect on the co-ordination chemical behaviour of trf.

In previous publications [15,16] on the complex-forming properties of histidine-containing dipeptides, we obtained new data confirming that the complex-forming conditions are influenced considerably by the imidazole side-chain of the dipeptides in comparison to glycylglycine. Depending on the relative positions of the amino, peptide amide, and imidazole-N^3 nitrogen donor atoms, however, the effects are observed in different ways. These differences and the conclusions on the copper(II) complexes of GlyHisGly and GlyGlyHis (and mainly the formation of the polynuclear and the bis complexes) justify the extension of the investigations to these tripeptides. This is all the more necessary since the uncertain conclusions stem from the differing findings on the mixed-ligand complexes. We have also performed equilibrium and spectral studies on the copper(II) complexes of L-pyroglutamyl-L-histidine methyl ester, a dipeptide analogue of trf.

Experimental

The tripeptides GlyHisGly and GlyGlyHis used were products of Sigma and Serva. L-Pyroglutamyl-L-histidyl-L-prolinamide (trf) and L-pyroglutamyl-L-histidine methyl ester (pghme) were provided by Gedeon Richter Pharmaceutical Works (Budapest). Other compounds were Reanal products of the highest analytical purity.

pH-Metric studies on the parent complexes were carried out at concentrations of $(1—2) \times 10^{-3}$ mol dm^{-3} and metal ion : ligand ratios of 1 : 1—1 : 5. All measurements were carried out at 25 °C and at an ionic strength of 0.2 mol dm^{-3} (KCl). Nitrogen was bubbled through the solutions to ensure the absence of oxygen. An inert atmosphere was particularly

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Table. Stability constants ($\log \beta_{pqr}$) of copper(II) complexes of histidine-containing peptides: $T = 298$ K, $I = 0.2$ mol dm^{-3} KCl, $pM + qA + rH \rightleftharpoons M_pA_qH_r$; $\beta_{pqr} = [M_pA_qH_r]/[M]^p[A]^q[H]^r$

	log β_{pqr}			
	GlyHisGly	GlyGlyHis	trf	pghme
HA	7.98	7.96	6.30	6.33
H_2A^+	14.33	14.60	—	—
H_3A^{2+}	17.05	17.52	—	—
$[CuA]^+$	9.05	—	3.64	4.11
$[CuA_2]$	15.72	—	—	—
$[CuAH_{-1}]$	5.46	—	—	—
$[CuA_2H_{-1}]^-$	8.36	—	—	—
$[CuAH_{-2}]^-$	—	−1.73	−7.67	−6.98

important for GlyGlyHis, for metal ions induce its oxidative decarboxylation.[17]

pH-Metric measurements employed a Radiometer pHM 64 pH-meter, G202B glass and K401 calomel electrodes, and an ABU 13 automatic burette. The titration method and the procedure for computer evaluation of the data have been described earlier.[18]

Spectrophotometric measurements were made in the visible region with a Beckman Acta MIV instrument, using 5-cm cells. The solution concentrations were the same as those in the pH-metric studies.

A calorimetric study of the copper(II)–trf system was made on 25-cm^3 samples with a concentration of 0.001 mol dm^{-3}, using a LKB 8700-2 reaction and solution calorimeter, as described previously.[19]

Results

The pH-metric titration curves for copper(II)–GlyHisGly are identical to those for copper(II)–GlyHis,[15] indicating that only one of the peptide amide groups is deprotonated and takes part in the complex formation. Accordingly, the pH-metric data for the interval $3.5 \leqslant pH \leqslant 8.5$ could be evaluated satisfactorily by assuming the presence of the complexes shown in the Table (which also contains data on the other systems examined in this work). The relevant concentration distribution curves are presented in Figure 1.

It is clear from Figure 1 that $[CuAH_{-1}]$ is the predominant species in the copper(II)–GlyHisGly system. Co-ordination in this complex involves the amino, the deprotonated peptide amide, and the imidazole-N^3 nitrogens.[2] However, assuming the presence of polynuclear complexes proposed by Österberg and Sjöberg[3] did not improve the accuracy of the evaluation. Thus, it appears probable that in dilute solutions the concentrations of polynuclear species are at most negligible. The pK value for deprotonation of the peptide amide group (pK = 3.59) is lower than that for GlyHis (pK = 4.15).[15] Since the amino and imidazole pK values for the free ligand are also lower, this decrease can presumably be explained by the peptide-bonded glycine in the ' side-chain ' being a weaker electron donor than the carboxylate group.

Figure 1 reveals that the complexes $[CuA_2]$ and $[CuA_2H_{-1}]^-$ are present only at higher pH, and in low concentration. The pK value shows that their formation is accompanied by only a slight pH effect, *i.e.* the accuracy of the evaluation of the data is not influenced appreciably if the bis complexes are ignored. To confirm that the bis complexes are not ' products ' of the computer evaluation, spectrophotometric measurements were carried out. The spectra are shown in Figure 2.

Curve (1) in Figure 2 depicts the spectrum obtained in a copper(II) : GlyHisGly = 1 : 1 solution after the complex

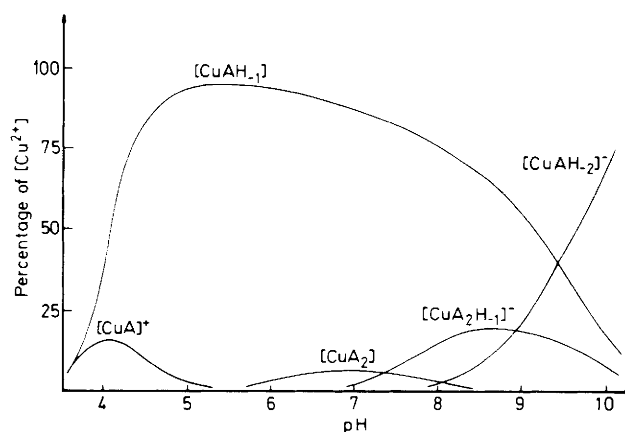

Figure 1. Concentration distribution of the complexes formed in the copper(II)–GlyHisGly system as a function of pH: $c_{Cu} = 6.25 \times 10^{-4}$ mol dm^{-3}, $c_A = 0.001$ mol dm^{-3}

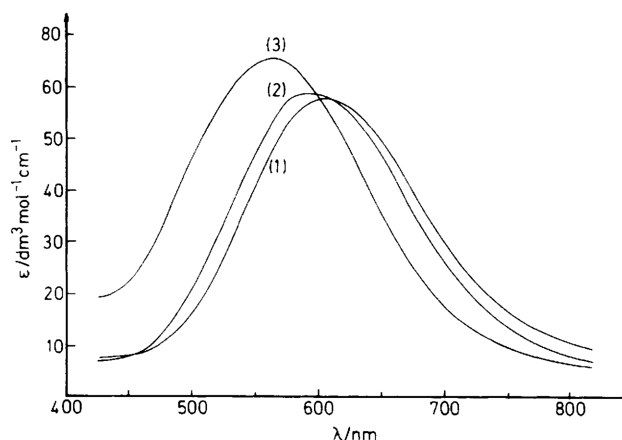

Figure 2. Absorption spectra of copper(II)–GlyHisGly solutions with various compositions: (1) $c_{Cu} = c_A = 0.001$ mol dm^{-3}, pH = 6; (2) $c_{Cu} = 3.33 \times 10^{-4}$, $c_A = 0.001$ mol dm^{-3}, pH = 8; and (3) $c_{Cu} = c_A = 0.001$ mol dm^{-3}, pH = 11.0

$[CuAH_{-1}]$ is formed in maximum amount ($\lambda_{max.} = 605$ nm, $\varepsilon = 58.0$ dm^3 mol^{-1} cm^{-1}). Curve (2) is the spectrum at pH = 8 in a copper(II)–GlyHisGly = 1 : 3 solution. Similarly as found for GlyHis,[15] the slight blue shift ($\lambda_{max.} = 590$ nm) is indicative of the co-ordination of additional N atoms, *i.e.* of the formation of bis complexes. Curve (3) is the spectrum for a 1 : 1 solution, registered at pH ~ 11 after the blue shift (begins at pH ~ 8) becomes unchanged. In agreement with the results of Aiba *et al.*,[6] a new base-consuming process begins in this interval. With the exclusion of the hydrolysis of the complexes, the considerable blue shift suggests the deprotonation of the imidazole N^{1}H. Similarly as for GlyHis,[7] polynuclear complexes are then formed through the co-ordination of these nitrogen donor atoms. Although an accurate composition cannot be determined from the equilibrium measurement data for these dilute solutions, a value of pK = 9.5 was calculated for $[CuAH_{-2}]^-$.

The pH-metric titration curves on the copper(II)–GlyGlyHis system show that the deprotonation of two peptide amide groups is to be considered in the complex formation. Accordingly, the experimental data can be described well on the assumption of only a single complex species, $[CuAH_{-2}]^-$. (The data are to be found in Table 1.) This result is not in

agreement with the findings of some authors, who also assumed the formation of bis complexes.[10,11] However, it does agree with other earlier results [6,8,9] and with findings from a structural study on the copper(II) complex of glycylglycylhistidine-N-methylamide.[20]

Under the concentration conditions applied in the measurements the quantity of the non-deprotonated 1 : 1 complex is not appreciable in the case of GlyGlyHis, presumably because of the larger size of the molecule and the higher stability of $[CuAH_{-2}]^-$. The spectrophotometric studies clearly demonstrated that bis complexes cannot be present in measurable concentrations either. Following deprotonation of the peptide amide groups, a well defined band gradually appears ($\lambda_{max.}$ = 522 nm, ε = 112 $dm^3 mol^{-1} cm^{-1}$); its development conforms with the $[CuAH_{-2}]^-$ concentration distribution calculated from the pH-metric data. The shape of the spectrum and the molar absorbance do not change when GlyGlyHis is in excess, which excludes the possibility of the formation of new species. From n.m.r. examinations, Kuroda and Aiba [21] likewise concluded that only the presence of a 1 : 1 complex need be considered even in a solution with a copper(II) : GlyGlyHis ratio of 1 : 50. At the same time, their results suggest that in solutions containing a high ligand excess, non-deprotonated 1 : 1 complexes might also be present in very low concentration below pH ~ 6.

N-Acetyl-L-histidine can be regarded as an analogue of GlyGlyHis in terms of binding. The results obtained for the copper(II)–*N*-acetyl-L-histidine system did not support the co-ordination of GlyGlyHis *via* the imidazole-N^3 and neighbouring deprotonated peptide amide nitrogen, and hence formation of complexes of 1 : 2 composition. [Deprotonation and co-ordination of the amide is disputed in the copper(II)–*N*-acetyl-L-histidine system, but it can occur at most in the interval pH > 10.[1,22]]

The spectrophotometric measurements confirm the formation of $[CuAH_{-2}]^-$ almost exclusively in the copper(II)–GlyGlyHis–histidine system. The absorption spectra were measured as a function of the histidine concentration at pH = 8.3 in solutions with a copper(II) : GlyGlyHis ratio of 1 : 1. Histidine (HB) led to a slight decrease in the band at 522 nm, while the absorption increased at higher wavelengths (after the 585 nm isosbestic point). The occurrence of the isosbestic point is indicative of the equilibrium $[CuAH_{-2}]^- + 2B^- + 2H^+ \rightleftharpoons [CuB_2] + A^-$. However, the spectral changes were not very great even at a five-fold histidine excess, and this equilibrium is therefore shifted strongly in the direction of the lower arrow. Thus, the experimental results agree well with the findings of Sakurai and Nakahara.[8] Accordingly, the mixed-ligand complexes cannot be present in measurable concentration in the range of existence of $[CuAH_{-2}]^-$ (pH > 6).

In a study of the copper(II)–GlyGlyHis system, Aiba *et al.*[6] described a further proton-consuming process in basic medium (p*K* = 10.69) which they interpreted in terms of deprotonation of the imidazole-N^1H moiety. With the exclusion of oxidative decarboxylation,[17] our measurements in an inert atmosphere suggest that a similar process can occur only at higher pH values (p*K* ~ 12). This p*K* value is similar to that for the copper(II)–histidine parent complex,[23] and thus deprotonation of the imidazole-N^1H without co-ordination is probable.

The results of the pH-metric equilibrium studies on trf and pghme agree will with the data found by Kozlowska *et al.*[14] The stability constants in the Table show that the two ligands behave similarly. In both cases the predominant complex species is $[CuAH_{-2}]^-$. Its formation can be explained by the participation of the deprotonated pyroglutamyl and the neighbouring peptide amide nitrogens in the co-ordination, together with the imidazole-N^3 nitrogens. Besides trf, Kozlowska *et al.*[14] also studied the copper(II) complex of pyroglutamyl-L-histidine, and found a value of −8.29 for log β_{11-2} (pK_{NH_3} = 7.18). The smaller p*K* values for pghme and its copper(II) complexes can presumably be explained by the weaker electron repulsion of the side-chain. (An analogous change could be observed in the comparison of GlyHis and GlyHisGly.)

Co-ordination *via* the three nitrogen donor atoms in $[CuAH_{-2}]^-$ means that the copper(II) ion has a free co-ordination site. As for GlyHis and GlyHisGly, in principle this provides a possibility for the formation of bis complexes. However, the small p*K* values of the ligands mean that the pH effect in the formation of $[CuA_2H_{-2}]^{2-}$ is barely noticeable. Therefore to settle this question spectrophotometric and calorimetric measurements were carried out.

The spectrophotometric investigations were performed in the same way as described for GlyHisGly. In the presence of a ligand excess, however, a blue shift was not observed at 6 ⩽ pH ⩽ 10; instead, the absorption band characteristic of $[CuAH_{-2}]^-$ was found at all ratios (for trf, $\lambda_{max.}$ = 585 nm; ε = 73 $dm^3 mol^{-1} cm^{-1}$; for pghme, $\lambda_{max.}$ = 585 nm; ε = 75 $dm^3 mol^{-1} cm^{-1}$).

In the calorimetric examination we set out to measure the reaction heat between a solution with a copper(II) : trf ratio of 1 : 1 at pH = 8 and 1 equivalent of trf excess. No heat effect was observed in the process, and thus the formation of bis complexes could be excluded on the basis of both the calorimetric and the previous spectrophotometric measurements. This difference compared to GlyHis and GlyHisGly can presumably be attributed to the greater spatial requirements of the trf molecule.

At pH > 10, a gradual blue shift may be observed in the spectra of copper(II)–trf [and copper(II)–pghme] solutions; this shows up as a further base-consuming process in the pH-metric titration curves. Due to the low concentrations applied the p*K* value can only be determined approximately, and it is uncertain whether the species formed is the $[CuAH_{-3}]^{2-}$ monomer or a polymeric complex similar in nature to that obtained with GlyHis. The p*K* values calculated for the formation of $[CuAH_{-3}]^{2-}$ are 10.5 ± 0.2 for trf, and 10.3 ± 0.2 for pghme. At pH ~ 12 the values of $\lambda_{max.}$ become constant at 560 nm, and thus ionization of the imidazole N^1H and participation of this nitrogen in the bonding is probable.

Conclusions

Together with previously published observations on histidine-containing dipeptides,[15] the results obtained in this work show that the histidine molecule incorporated in the peptide chain is an essential factor in determining the co-ordination chemical behaviour of small peptides.

The tripeptide GlyHisGly behaves similarly to the dipeptide GlyHis. For both ligands the predominant complex species is $[CuAH_{-1}]$, which is partially converted to the bis complex $[CuA_2H_{-1}]^-$ {or $[CuA_2] \equiv [Cu(AH_{-1})(HA)]$} in a ligand excess. The imidazole side-chain of the histidine in position 2 relative to the terminal amino-group therefore blocks co-ordination of further parts of the peptide molecule. The same phenomenon may be observed with trf, the complex-forming properties of which can be regarded as identical with those of L-pyroglutamyl-L-histidine (or its methyl ester), *i.e.* the co-ordination chemical effect of the prolinamide is negligible. In connection with this it should be noted that the prolinamide side-chain does not exhibit appreciable co-ordination properties in other peptides which do not contain histidine.[24] In the case of trf, therefore, the bonding mode in $[CuAH_{-2}]^-$ is a consequence

of the joint effects of the strongly co-ordinating histidine and the ' inactive ' prolinamide.

From the above considerations it is obvious that in the tripeptide GlyGlyHis the bonding involving the participation of the four nitrogen atoms results in the high stability of the complex $[CuAH_{-2}]^-$. In this species the copper(II) ion does not possess a free equatorial co-ordination site, and thus the tendencies to form bis and mixed-ligand complexes can be neglected. In accordance with the high stability of $[CuAH_{-2}]^-$, the intermediate ' singly deprotonated ' complex $[CuAH_{-1}]$ is not formed in measurable concentration. This means that in the case of GlyGlyHis the peptide amide groups undergo deprotonation in one overall process, in a ' co-operative manner '.[1]

Special mention should be made of the deprotonation and subsequent co-ordination of the N^1H group of the imidazole side-chain in the peptides. For GlyHis and GlyHisGly, in the complexes $[CuAH_{-1}]$ with a free co-ordination site the deprotonation proceeds very easily due to the possibility of co-ordination of the nitrogen atom (for GlyHisGly, $pK = 9.5$). A similar process also occurs for trf and pghme, however, the larger spatial requirements of the ligands mean that the corresponding pK values are higher ($pK = 10.5$ for trf). In contrast, there is no possibility for further co-ordination in the complex $[CuAH_{-2}]^-$ in the case of GlyGlyHis; thus, the deprotonation constant is larger ($pK \sim 12.0$), and falls in the range for the histidine parent complexes of type $[CuA_2]$.[23]

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