Stability Constants of Complexes of Copper(II) and Zinc(II) Ions with Histidylhistidine

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Equilibrium constants at 37 °C and I = 0.15 mol dm^{-3} (K[NO_3]) are reported for copper(II) and zinc(II) complexes of histidylhistidine (HL) from pH-titration data, using a range of metal-ion and ligand concentrations. Binuclear complexes are the major species in all the solutions. In the presence of histidine (HA), zinc(II) forms a mixed-ligand species, [ZnA(L)].

ALTHOUGH histidyl residues (His-) are important as metal-ion binding sites in proteins, only rarely do two His- residues occur consecutively in the same protein chain. At present the only known examples are in the β chain of human haemoglobin [1] and in sperm-whale myoglobin.[2] In both cases, however, *X*-ray structure determinations indicate rigidity in these, as in other, portions of the molecules, and the spaces around the two His- residues are filled by other parts of the protein chains. These effects would be expected to hinder any mutually co-operative metal binding by the two His- residues and, in fact, no metal binding by these portions has been reported. In order to examine the possibility that metal binding by unhindered adjoining His- in a simple peptide chain might be in some way anomalous, we have determined the stability constants of copper(II) and zinc(II) complexes of histidylhistidine (His-His), using the potentiometric technique described earlier.[3,4]

Quantitative information on the stability of metal complexes of His-His is meagre. At pH 5.2, with the ligand in excess, His-His binds copper(II) ion (1.4×10^{-3} mol dm^{-3}) more strongly than does histidine (His).[5] Stepwise pK_a values for the loss of three protons from 1 : 1 mixtures of His-His and Cu^{II} and Ni^{II} have been reported.[6] On the assumption that only two complexes are formed, titration curves for a two-fold variation in peptide concentration were interpreted to yield equilibrium constants for a 1 : 1 copper(II)–histidylhistidine complex from which a proton could be ionised.[7] However, as Cu^{I} forms binuclear complexes with His-His,[8] it is possible that other metal ions behave similarly. For this reason it is important when carrying out potentiometric titrations to cover an adequate range of metal-ion and ligand concentrations.

EXPERIMENTAL

Materials.—DL-Histidyl-DL-histidine (His-His) (Nutritional Biochemical Corporation, U.S.A.) dried *in vacuo* over P_2O_5, was checked for purity by elemental analysis and by potentiometric titration. Solutions of this peptide were prepared immediately before use in boiled-out glass-distilled water. All other reagents were as described earlier.[3,4]

Procedure.—Potentiometric titrations and the resulting computations followed established procedures [3,4] to yield pK_a and stability-constant values at 37 °C and I = 0.15

[1] W. Konigsberg, J. Goldstein, and R. J. Hill, *J. Biol. Chem.*, 1963, **238**, 2028.
[2] A. B. Edmundson, *Nature*, 1965, **205**, 883.
[3] R. P. Agarwal and D. D. Perrin, *J.C.S. Dalton*, 1975, 268.
[4] R. P. Agarwal and D. D. Perrin, *J.C.S. Dalton*, 1975, 1045.
[5] G. Wolff, S. Fallab, and H. Erlenmeyer, *Experientia*, 1965, **11**, 440.
[6] R. B. Martin and J. T. Edsall, *J. Amer. Chem. Soc.*, 1960, **82**, 1107.
[7] M. A. Doran, S. Chaberek, and A. E. Martell, *J. Amer. Chem. Soc.*, 1964, **86**, 2129.
[8] T. Kaden and A. Zuberbühler, *Helv. Chim. Acta*, 1966, **49**, 2189.

mol dm^{-3} ($K[NO_3]$). Metal-ion concentrations were varied over a five-fold range (1×10^{-3}—5×10^{-3} mol dm^{-3}) for two sets of solutions in which the ligand-to-metal ion ratios were 1 : 1 and 2 : 1. The pH range covered in the titrations were 3.2—7.8 for copper and 4.7—6.9 for zinc. In solutions containing equimolar concentrations of zinc ion and ligand, precipitation occurred at pH *ca.* 6.7 ± 0.2. All potentiometric-titration data are in Supplementary Publication No. SUP 21574 (13 pp.).*

RESULTS AND DISCUSSION

The experimental pK_a values (for proton loss) for histidylhistidine were assigned as follows: 2.23, carboxyl group; 5.15, imidazole group [*cf.* 5.39 for (His-Gly) [3]]; 6.55, imidazole group (*cf.* 6.61 for (Gly-His) [3]]; and 7.52, protonated α-amino-group (*cf.* 7.15 for His-Gly [3]).

By analogy with other dipeptides, Cu^{II} and His-His would be expected to form a series of 1 : 1 and 1 : 2 metal–ligand complexes in which the ligand varied from $[H_2L]^+$ to $[L - H]^{2-}$, where L is the peptide anion. Computer-based analysis of the titration data between pH 3 and 6 for 1 : 2 metal–ligand mixtures afforded values for these stability constants that showed a trend with metal-ion concentration. This trend, which was much more evident at higher pH values, was not present when binuclear complexes were assumed to be formed. On this assumption, the eight stability constants and three pK_a values given in Table 1 were obtained. They

TABLE 1

Equilibrium constants and their standard deviations for copper(II) complexes of histidylhistidine (HL) at 37 °C and I = 0.15 mol dm^{-3} ($K[NO_3]$)

Equilibrium	log K
$Cu^{2+} + [H_2L]^+ \rightleftharpoons [Cu(H_2L)]^{3+}$	3.37 ± 0.03
$Cu^{2+} + HL \rightleftharpoons [Cu(HL)]^{2+}$	7.67 ± 0.02
$Cu^{2+} + L^- \rightleftharpoons [CuL]^+$	10.82 ± 0.01
$Cu^{2+} + [H_2L]^+ + HL \rightleftharpoons [Cu(H_3L_2)]^{3+}$	10.66 ± 0.02
$Cu^{2+} + 2HL \rightleftharpoons [Cu(H_2L_2)]^{2+}$	13.00 ± 0.02
$Cu^{2+} + HL + L^- \rightleftharpoons [Cu(HL_2)]^+$	14.56 ± 0.01
$Cu^{2+} + 2L^- \rightleftharpoons [CuL_2]$	15.44 ± 0.01
$2[CuL]^+ \rightleftharpoons [Cu_2L_2]^{2+}$	1.65 ± 0.06
	pK_a
$[CuL_2] \rightleftharpoons [CuL(L - H)]^- + H^+$	7.67 ± 0.01
$[Cu_2L_2]^{2+} \rightleftharpoons [Cu_2L(L - H)]^+ + H^+$	4.96 ± 0.07
$[Cu_2L(L - H)]^+ \rightleftharpoons [Cu_2(L - H)_2] + H^+$	5.94 ± 0.02

Ligand pK_a values are 2.23 ± 0.05, 5.15 ± 0.03, 6.55 ± 0.02, and 7.52 ± 0.01.

reproduced the five titration curves for 1 : 1 and 1 : 2 metal–ligand ratios (151 points) with a standard deviation in titre of 0.0010 cm^3. The following species accounted for more than 20% of the total copper species during parts of one or more titrations: $[Cu(HL)]^{2+}$; $[CuL]^+$; $[Cu(HL)_2]^{2+}$; $[Cu(HL_2)]^+$; $[CuL_2]$; $[CuL(L - H)]^-$; $[Cu_2L(L - H)]^+$; and $[Cu_2(L - H)_2]$. The species $[Cu(L - H)]$ and $[Cu_2(HL_2)]^{3+}$ could be neglected.

The copper(II)–histidylhistidine complex $[Cu(H_2L)]^{3+}$ (log K 3.37) is assumed to be protonated on the α-amino- and a histidyl imidazole nitrogen atom, and hence to have the same metal-binding sites as in the $[Cu(HL)]^{2+}$ complex of copper(II)–glycylhistidine (log K 4.28).[3] (The alternative $[Cu(HL)]^{2+}$ complex of copper(II)–histidylglycine is unknown.) The difference of about one logarithm unit between the stability constants is attributed to the effect of the additional positive charge on $[Cu(H_2L)]^{3+}$. Similar comments apply to the complexes $[Cu(HL)]^{2+}$ for copper(II)–histidylhistidine (log K 7.67) and $[CuL]^+$ for copper(II)–glycylhistidine (log K 8.68).[3] However, the stability constant of the copper(II)–histidylhistidine complex $[CuL]^+$ (log K 10.82) is much greater than for the copper(II)–glycylhistidine or –histidylglycine complex (log K 8.02 [3]), and indicates that both imidazole groups of His-His are involved in the chelation to Cu^{II}.

The non-planarity of peptide, imidazole, and amine nitrogen atoms of His-Gly co-ordinated to Cu^{2+} [3,9] makes it very unlikely that both of the imidazole groups of His-His lie in the same plane as the other atoms bonded to Cu^{II}. A study of atomic models supports this conclusion and suggests that only the peptide, imidazole, and amine nitrogen atoms corresponding to those of Gly-His lie in a plane around Cu^{2+}, and the (amino-terminal) imidazole group is located axially. An analogous non-planar structure has been suggested [9] for histidylglycinatocopper(II). Non-participation of this amino-terminal imidazole group in bonding to copper in bis(histidylhistidinato)copper(II), $[CuL_2]$, would explain the near identity of the stability-constant values for the $[CuL_2]$ complexes of Gly-His (log K 15.41 [3]) and His-His (log K 15.44). Protonation of one or both of the non-bonded imidazole nitrogen atoms gives the complex $[Cu(HL_2)]^+$ or $[Cu(L_2)_2]^{2+}$, with somewhat lower stability constants because of electrostatic interactions.

Although the constants for the dimerisation reactions (1) of His-Gly (log K −9.15) and His-His (−9.25) are

$$2[CuL]^+ \rightleftharpoons [Cu_2(L - H)_2] + 2H^+ \qquad (1)$$

similar, spectral studies suggest that the complexes differ significantly in structure. In aqueous solutions, copper(II) complexes in which the metal is bonded through oxygen and nitrogen atoms show a broad absorption band with a maximum in the range 560—800 nm, depending on the number of nitrogen atoms. Some appropriate examples are given in Table 2. The similarity of the maxima for the $[Cu_2L_2(OH)_2]$ complexes of histamine (612 nm) and His-Gly (613 nm) supports the belief [3] that the latter is also a hydroxo-bridged species in which each copper ion is bonded to an imidazole, and an amine, nitrogen atom and to two (shared) hydroxyl groups. On the other hand, the maximum (581 nm) for the $[Cu_2(L - H)_2]$ complex of His-His suggests that each copper ion is co-ordinated to three or four nitrogen atoms. A possible structure, which is consistent with atomic models and with crystallographic studies [10] of

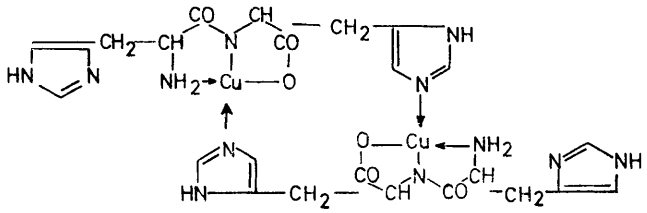
* For details see Notice to Authors No. 7, *J.C.S. Dalton*, 1975, Index issue.

[9] G. F. Bryce, R. W. Roeske, and F. R. N. Gurd, *J. Biol. Chem.*, 1965, **240**, 3837.

[10] H. C. Freeman and J. T. Szymanski, *Acta Cryst.*, 1967, **22**, 406.

dimeric copper(II)–carnosine dihydrate, is shown in (I). In such a structure the two pK_a values (4.86 and 5.94) would be for the ionisation of peptide hydrogen atoms and their sum is comparable with the combined pK_a values (10.60 [3]) for the carnosine complex.

The present study illustrates the need to use a wide range of ligand and metal-ion concentrations in seeking to obtain stability constants of metal complexes. Thus, earlier workers [7] analysed the pH-titration curve for an equimolar solution, *ca.* 2.5×10^{-3} mol dm^{-3} in copper(II) nitrate and histidylhistidine, by assuming the formation only of $[CuL]^+$ and $[Cu(L - H)]$. Using all our titration data we were unable to refine an equilibrium constant for $[Cu(L - H)]$; the computer estimate of the constant decreased progressively with successive cycles of iteration and became negligible. This may be



(I)

because $[CuL]^+$ represents more than one species and may include complexes of the type $[CuH(L - H)]^+$, in which the proton is removed from the peptide nitrogen but one of the imidazole or amino-nitrogen atoms is also protonated. From the constants given in Table 1 we calculated the pH-composition profile of a 2.5×10^{-3} mol dm^{-3} copper(II) nitrate–histidylhistidine solution (Figure). The complexity of the system is obvious. At pH 5.8 the dimeric species $[Cu_2L(L - H)]^+$ and $[Cu_2(L - H)_2]$ are major components. Near pH 7, $[Cu_2(L - H)_2]$ is *ca.* 90% of the total copper, whereas at pH 4 *ca.* 50% of the copper is present as $[Cu(HL)]^{2+}$.

A similar analysis of six titration curves for 1 : 1 and 1 : 2 zinc(II) : ligand ratios (142 points, pH range 4.7—6.9) gave the constants listed in Table 3 for zinc(II)–histidylhistidine complexes. These constants reproduced the titration curves with a standard deviation in titre of 0.0019 cm^3. Like the corresponding copper complexes, the zinc(II)–histidylhistidine complexes $[ZnL]^+$ and $[ZnL_2]$ (log K_1 4.97, log β_2 9.61) are more stable than the zinc(II)–glycylhistidine (log K_1 3.65,[4] log β_2 6.89 [4]) or –histidylglycine complexes (log K_1 4.25,[4] log β_2 8.46 [4]). The assumption that the enhanced stability is due to metal binding by both imidazole groups is difficult to sustain for the bis complex. If the co-ordination number for Zn^{II} is not to exceed six, chelation through nitrogen atoms of both imidazole groups in His-His would require the formation of at least a seven-membered chelate ring and this is regarded as lacking stability.

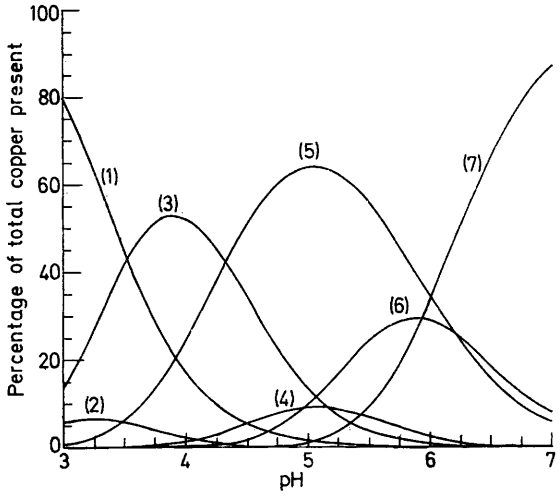
Assignment of structures to other complexes is based on quantitative comparisons with published constants and on the assumption that in its bonding to Zn^{II} protonated His-His behaves as an imidazolinium

TABLE 2

Absorption maxima of some copper(II) complexes in aqueous solution

Complex	Ligand	Number of N atoms per Cu	$\lambda_{max.}$/nm	Ref.
Cu^{2+}(aq)	water	0	*ca.* 800	
$[CuL_2]$	α-amino-acid	2	*ca.* 620	*a*
$[Cu_2L_2(OH)_2]$	histamine [b]	2	612	
$[Cu_2L_2(OH)_2]$	His-Gly [b]	2	613	
$[Cu(L - H)]$	Gly-His [b]	3	600	
$[Cu(L - 2H)]^-$	Ac-Gly-Gly-His	3	590	*c*
$[CuL_4]$	imidazole	4	596	*d*
$[CuL_2]$	histamine	4	593	
$[CuL]$	3,6-diazaoctane-1,8-diamine	4	576	*e*
$[CuL_2]$	ethylenediamine	4	565	*e*
$[Cu_2(L - H)_2]$	His-His [b]	?	581	

[a] R. D. Gillard, H. M. Irving, R. M. Parkins, N. C. Payne, and L. D. Pettit, *J. Chem. Soc.* (*A*), 1966, 1159. [b] 1:1 Metal: ligand solution, pH 8. [c] G. F. Bryce and F. R. N. Gurd, *J. Biol. Chem.*, 1966, **241**, 122. [d] J. T. Edsall, G. Felsenfeld, D. S. Goodman, and F. R. N. Gurd, *J. Amer. Chem. Soc.*, 1954, **76**, 3054. [e] M. Kato, *Z. phys. Chem.* (*Frankfurt*), **23**, 375, 391.



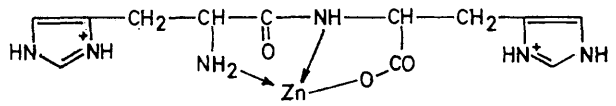
Variation with pH of the computed composition of a solution of histidylhistidine (2.5×10^{-3} mol dm^{-3}) and Cu^{2+} (2.5×10^{-3} mol dm^{-3}), as a percentage of the total copper present: (1), free Cu^{2+}; (2), $[Cu(H_2L)]^{3+}$; (3), $[Cu(HL)]^{2+}$; (4), $[Cu_2L_2]$; (5), $[CuL]^+$; (6), $[Cu_2L(L - H)]^+$; and (7), $[Cu_2(L - H)_2]$

TABLE 3

Equilibrium constants and their standard deviations for zinc(II) complexes of histidylhistidine (HL) at 37 °C and I = 0.15 mol dm^{-3} (K[NO$_3$])

Equilibrium	log K
$Zn^{2+} + [H_2L]^+ \rightleftharpoons [Zn(H_2L)]^{3+}$	2.31 ± 0.05
$Zn^{2+} + HL \rightleftharpoons [Zn(HL)]^{2+}$	4.06 ± 0.02
$Zn^{2+} + L^- \rightleftharpoons [ZnL]^+$	4.97 ± 0.05
$Zn^{2+} + 2HL \rightleftharpoons [Zn(H_2L_2)]^{2+}$	7.53 ± 0.03
$Zn^{2+} + HL + L^- \rightleftharpoons [Zn(HL_2)]^+$	8.99 ± 0.02
$Zn^{2+} + 2L^- \rightleftharpoons [ZnL_2]$	9.61 ± 0.02
$[Zn(HL)]^{2+} + [ZnL]^+ \rightleftharpoons [Zn_2(HL_2)]^{3+}$	2.96 ± 0.09
$2[ZnL]^+ \rightleftharpoons [Zn_2L_2]^{2+}$	3.37 ± 0.11

derivative of Gly-His or His-Gly. Thus, by analogy with the $[Zn(HL)]^{2+}$ complex of His-Gly,[4] the metal ion in the $[Zn(H_2L)]^{3+}$ complex of His-His is probably chelated through a carboxyl oxygen and the peptide and amino-nitrogen atoms, as in (II), and the imidazole



(II)

groups are protonated. Stability constants (log K_1 2.31 for $[Zn(H_2L)]^{3+}$ of His-His, 2.37 for $[Zn(HL)]^{2+}$ of His-Gly [4]) are consistent with this view. The agreement of the log K_1 and log β_2 values for the $[Zn(HL)]^{2+}$ and $[Zn(HL)_2]^{2+}$ complexes of His-His (4.06 and 7.53) and for the $[ZnL]^+$ and $[ZnL_2]$ complexes of Gly-His (3.65 and 6.89 [4]) and His-Gly (4.25 and 8.46 [4]) is explicable in the same way. Likewise, the near identity of the dimerisation constants ($2[ZnL]^+ \rightleftharpoons [Zn_2L_2]^{2+}$) for the zinc(II) complexes of Gly-His (3.30 [4]) and His-His (3.37) suggests that their binuclear species are structurally similar. Although deprotonated species such as $[Zn_2L(L - H)]^+$ (pK_a 7.39 [4]) are formed in the zinc(II)–glycylhistidine system, they were not detected in the study with His-His, probably because the accessible pH range was too low.

The pH-titration curves of solutions containing Cu^{2+} and mixtures of His (HA) and His-His were not significantly different from those computed from the pK_a values of the ligands and the stability constants of the individual metal chelates. With Zn^{II},[11] on the other hand, the curves differed considerably, indicating the formation of mixed-ligand complexes. Best fit of the

$$Zn^{2+} + A^- + L^- \rightleftharpoons [ZnA(L)], \log K\ 10.31 \quad (2)$$

experimental data required postulation of two species [equations (2) and (3)]. Agreement to within a standard deviation in titre of 0.0020 cm^3 was found for five computed and experimental titration curves (75 points) over the pH range 6.1—7.7. These species appear to be

$$2Zn^{2+} + A^- + L^- \rightleftharpoons [Zn_2A(L - H)]^+ + H^+, \quad \log K\ 8.84 \quad (3)$$

strictly analogous to the [ZnA(L)] formed by His-Gly (log K 10.12 [4]), [CoA(L)] formed by His-Gly (10.50 [4]) and Gly-His (9.36 [4]), and $[Zn_2A(L - H)]^+$ formed by Gly-His (10.04 [4]). The logarithm of the stability constant is not very different from the mean of log β_2 for $[Zn(His\text{-}His)_2]$ and $[Zn(His)_2]$, namely $\frac{1}{2}(9.61 + 11.69) = 10.65$.

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[11] D. D. Perrin and V. S. Sharma, *J. Chem. Soc.* (*A*), 1967, 724.