A Potentiometric and Spectroscopic Study of the Interaction of Ala–Ala–Asp–Ala with Cu(II)

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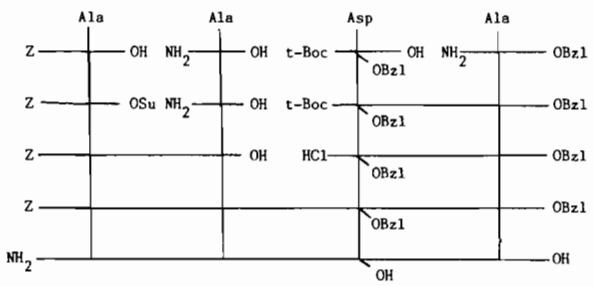
A recent study of the biologically active pentapeptide fragment of thymopoietin, Arg–Lys–Asp–Val–Tyr (H_3L) showed that the major complex formed with Cu(II) ions over the pH range of 5–9 is the [CuL] species which has three nitrogen atoms coordinated to the metal ion [1]. At higher pH two further protons can be ionized to give $[CuH_{-1}L]$ and $[CuH_{-2}L]$ species, but neither of these appear to involve four nitrogen coordination as would be expected for normal polypeptides [2]. This behaviour must be a result of the presence of the side-chain of the Asp residue, with the possibility of coordination through the terminal β-carboxylate group. We have recently shown that when Asp is in the second position in a peptide sequence the β-carboxylate coordinates strongly to Cu(II) ions [3]. To examine the effect of Asp in the third position, as is the case of the active fragment of thymopoietin, we have synthesised Ala–Ala–Asp–Ala (H_2L) and studied its interaction with Cu(II) ions potentiometrically and spectroscopically.

Experimental

Peptide Synthesis

Ala–Ala–Asp–Ala was synthesised by standard liquid phase methods as outlined in Scheme 1.



Scheme 1

The coupling reagents were dicyclohexylcarbodiimide (DCCI, Merck) and 1-hydroxybenzotriazole (HOBt, Aldrich). The t-butyloxycarbonyl (t-Boc) groups were cleaved by 4 mol dm^{-3} HCl–dioxan. The benzyloxycarbonyl (Z) and benzyl groups were removed by hydrogenolysis using 10% Pd/C in a methanol/acetic acid (90/10) mixture. The peptide was purified by gel filtration (Sephadex G10, eluent water). Sample purity was checked by paper chromatography (Whatman no. 1, eluent (%): water (30), pyridine (35) and butanol (35)) and by HPLC on bondapak C_{18} with an eluent gradient of water (with 0.05% trifluoroacetic acid)–methanol. Amino acid analysis gave the following results: aspartic acid (1.09), alanine (2.91).

Spectroscopic Studies

The metal ion sources were $Cu(ClO_4)_2 6H_2O$ (Fluka) and solutions with molar ratios of metal: peptide of 1:1 were used with concentrations of 0.002–0.003 mol dm^{-3}. Absorption spectra were measured on a Cary 219 spectrometer and circular dichroism (CD) spectra on a Mark III Jobin-Yvon dichrograph in the 200–800 nm region. All CD results are expressed in terms of $\Delta\epsilon = \epsilon_l - \epsilon_r$. Electron paramagnetic resonance (EPR) spectra were recorded on a Varian E102 spectrometer at liquid nitrogen temperature, diphenylpicrylhydrazine being used as a standard.

Potentiometric Studies

Stability constants for H^+ and Cu(II) complexes were calculated from titration curves carried out at 25 °C using total volumes of 1.5–2 cm^3. Alkali was added from a 0.1 or 0.25 cm^3 micrometer syringe which had been calibrated by both weight titration and the titration of standardized materials. Changes in pH were followed using a glass electrode calibrated in H^+ concentrations with $HClO_4$. 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 [4]. By means of this program it was possible to demonstrate the absence of acetate in the ligand solution. In all cases duplicate or triplicate titrations were carried out at Cu:L ratios of 1:1 and 1:2. The standard deviations quoted were computed by SUPERQUAD and refer to random errors only. They give, however, a good indication of the importance of the particular species in the equilibrium.

Results and Discussion

Hydrogen Ion Complexes

Ala–Ala–Asp–Ala (H_2L) has 3 protonation centres, a terminal carboxylate group, the β-carboxy-

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TABLE I. Formation Constants of H^+, Cu^{2+} Complexes at 25 °C and $I = 0.10$ mol dm^{-3} (KNO_3), with Standard Deviations on the Last Figure Given in Parentheses

H^+ Complexes	log values				
	β_{HL}	β_{H_2L}	β_{H_3L}	K_{H_2L}	K_{H_3L}
Ala–Ala–Asp–Ala	8.29(1)	12.51(1)	15.80(1)	4.22	3.29
Tetraalanine[a]	8.13(1)	11.65(1)		3.52	
Tetraglycine[b]	7.97	11.15		3.18	
Cu^{2+} complexes	log values				
	β_{CuL}	$\beta_{CuH_{-1}L}$	$\beta_{CuH_{-2}L}$	$\beta_{CuH_{-3}L}$	
Ala–Ala–Asp–Ala	5.36(8)	0.57(2)	−4.73(1)		
(stepwise)		4.79	5.30		
Arg–Lys–Asp–Val–Tyr[c]	7.68		−3.42	−11.10	
Tetraalanine[a]	4.77(5)	−0.45(1)	−8.09(1)	−17.33(2)	
(stepwise constants)		5.22	7.64	9.24	
Tetraglycine[b]	5.08	−0.42	−7.31	−16.60	

[a]Ref. 3. [b]Ref. 2. [c]Ref. 1. The ligand (assumed H_2L) contains two additional ionizable protons from the terminal NH_3- of −Lys− and the phenylic −OH of −Tyr. For the sake of comparison, these have been ignored when calculating the above constants.

TABLE II. Spectroscopic Data for Cu^{2+} Complexes of Ala–Ala–Asp–Ala

Technique	pH Range	Assignment	λ_{max} (nm) ($\Delta\epsilon$[a])
Absorption	5–11.5	d–d	548
CD	5–11.5	d–d	576(−0.42) B transition
		d–d	492 (+0.16) E transition
		N^-–Cu CT	306 (+0.59)
		NH_2–Cu CT	270(−2.15)
		CO_2^-–Cu CT	244 (+0.26) (weak)
		intraligand CT	228(−1.2)
EPR	5–11.5	$A_\parallel = 205$ G	$g_\parallel = 2.19$

[a]For the CD results, ϵ (dm^{-3} mol^{-1} cm).

late of the Asp residue and a terminal amino group. Protonation constants are given in Table I, where K_{HL} corresponds to protonation of the amino group (log $K = 8.29$) while the other stepwise constants (log $K = 4.22$ and 3.29) are for carboxylate protonations. These values are compatible with those for tetraalanine (tetraAla), which has one carboxylate less [3].

Copper(II) Complexes

Stability constants are also given in Table I, and the spectroscopic characteristics in Table II. The species distribution curves are given in Fig. 1. Only three complexes are formed in significant concentrations between pH 5 and 10, [CuL], [$CuH_{-1}L$] and [$CuH_{-2}L$] (charges omitted). Only one species could be detected spectroscopically. Throughout this pH range both carboxyl protons will be ionized whether the groups are coordinated or not. Hence the complexes can be compared directly with those for tetraAla or Ala–Asp–Ser–Gly as far as empirical formulae are concerned. Comparison with Arg–Lys–Asp–Val–Tyr is more difficult since this ligand has two additional potentially ionizable protons, one on the phenolate group (log $K = 9.75$) and the other on the lysyl amino group (log $K = 10.38$) [1]. If it is assumed that these protons are not ionized below pH 8 then the ligand can be also regarded as [H_2L], and the stability constants defined accordingly, as in Table I.

From Table I and Fig. 1 it is clear that Ala–Ala–Asp–Ala forms more stable complexes than does tetraAla. The [CuL] complex is still a minor species. It is normally bonded through the terminal amino nitrogen and the neighbouring carbonyl oxygen, but this may be supported by some β-carboxy-

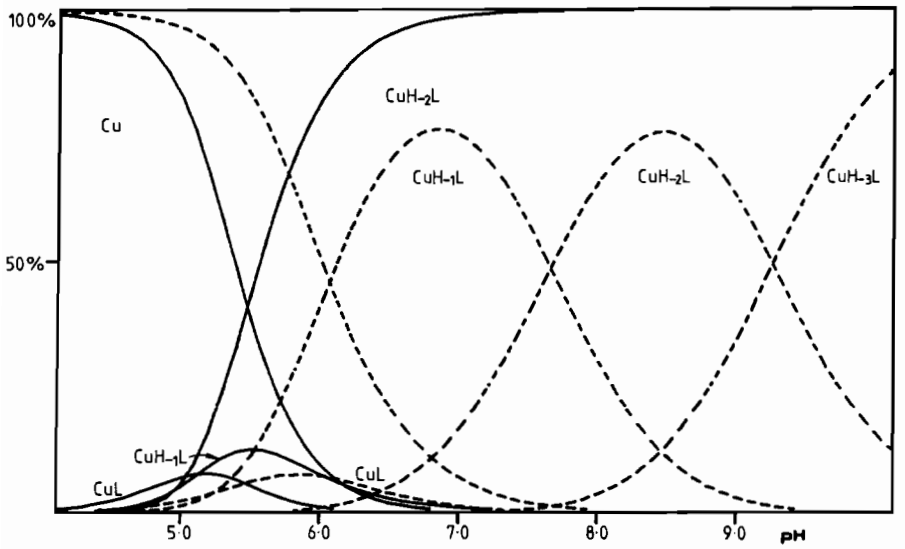

Fig. 1. Species distribution curves for the Cu(II) complexes of tetraalanine (dotted lines) and Ala–Ala–Asp–Ala (solid lines). Cu(II):peptide = 1:1, 0.001 mol dm^{-3}.

late coordination although the chelate ring would be abnormally large. Around pH 5, Cu(II) promotes ionization of the first peptide amide proton to give $[CuH_{-1}L]$. With tetraAla this is a significant species while with Ala–Ala–Asp–Ala it is comparatively unimportant and could not be detected unambiguously by spectroscopy (maximum concentration less than 25% in a 0.001 mol dm^{-3} solution, see Fig. 1). With Arg–Lys–Asp–Val–Tyr it was undetectable [1]. In spite of its low importance in the equilibrium with Ala–Ala–Asp–Ala, the complex is significantly more stable than with tetraAla (Δ log $\beta = 1.12$), again suggesting β-carboxylate interaction. The major difference between Ala–Ala–Asp–Ala and tetraAla is seen in the $[CuH_{-2}L]$ complex where Δ log β = 3.36. An even more dramatic stabilization is seen in the results for Arg–Lys–Asp–Val–Tyr (Δ log β = 5.67) [1]. This difference is further emphasised by the absence of a $[CuH_{-3}L]$ species with 4 nitrogen coordination, although it was searched for both potentiometrically and spectroscopically up to pH 11.5. With tetraAla it is a major species above pH 9 and is clearly an NNNN complex. Similarly with Arg–Lys–Asp–Val–Tyr no NNNN complexes were formed since deprotonation of the NNN complex ($[CuH_{-2}L]$ in Table I) was shown to be the result of ionization of the Tyr–OH and Lys–NH_3^+ protons [1].

The absence of NNNN complexes must be a result of the abnormal stability of the NNN coordinated $[CuH_{-2}L]$ complexes. These would be bonded through the terminal amino nitrogen and the amide nitrogens of the first and second peptide bonds. From this position the β-carboxylate oxygen of Asp could bond in the coordination plane forming a six

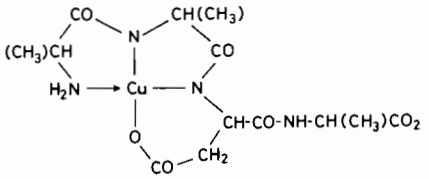

Fig. 2. The $[CuH_{-2}L]$ species with Ala–Ala–Asp–Ala.

membered chelate ring as shown in Fig. 2 so causing the enhanced stabilities found. The stability of this complex would naturally discourage ionization of the third peptide proton to form the normal NNNN complex. This conclusion is supported admirably by the spectroscopic results which are consistent with an NNN bonded complex over the entire pH range of 5–11.5. The absorption maximum for the d–d transition (548 nm) is slightly higher in energy than that normally observed for NNN coordination, but is compatible with that expected for NNNO bonding, using an Asp–carboxylate oxygen [5]. Likewise the CD spectra showed bands characteristic of both N^-–Cu and NH_2–Cu interaction [2], and of CO_2^-–Cu interaction [6]. The EPR spectrum was also characteristic of NNN coordination [7].

As a result of the above study it can also be assumed that the peculiar stability of the corresponding NNN complex with Arg–Lys–Asp–Val–Tyr is due to β-carboxylate interaction which is even more marked than with Ala–Ala–Asp–Ala for conformational reasons. Hence the β-carboxylate group actively participates in coordination to Cu(II) and does more than 'hinder' coordination of the fourth nitrogen [1].

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