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Co-ordinating properties of cyclopeptides. Thermodynamic and spectroscopic study on the formation of copper(II) complexes with cyclo(Gly-His)$_4$ and cyclo(Gly-His-Gly)$_2$ and their superoxide dismutase-like activity

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Two cyclopeptides, cyclo(Gly-His)$_4$ and cyclo(Gly-His-Gly)$_2$ were synthesized with the specific aim to form copper(II) complexes which are able to mimic the active site of superoxide dismutase. Proton and copper(II) complexes were thermodynamically characterized. The copper(II) complexes were also studied by means of optical and ESR spectroscopy to gain information on their structural features and by voltammetry to know about their redox ability. Moreover, the antioxidant activity of these complex species was tested against enzymatically generated superoxide radical. Depending on the pH value of the solution, definite complexes could be characterized, in particular $[Cu\{cyclo(Gly\text{-}His)_4\}]^{2+}$ and $[Cu\{cyclo(Gly\text{-}His)_4\}H_{-2}]$ and $[Cu\{cyclo(Gly\text{-}His\text{-}Gly)_2\}H_{-2}]$ are the main species, which were taken into consideration to assay their antioxidant catalytic activity. The ESR studies suggested that a four-nitrogen co-ordination by means of imidazole nitrogen atoms or deprotonated peptide nitrogen atoms forms the environment around copper. At the same co-ordination level, the redox properties of these compounds parallel their scavenging abilities against O_2^- which are lower than those of other copper(II) complexes previously tested. The $[Cu\{cyclo(Gly\text{-}His)_4\}]^{2+}$ complex showed higher redox potential and better catalytic ability than $[Cu\{cyclo(Gly\text{-}His)_4\}H_{-2}]$ and $[Cu\{cyclo(Gly\text{-}His\text{-}Gly)_2\}H_{-2}]$, which have roughly similar redox potentials and scavenging abilities.

Introduction

The production of reactive oxygen species such as superoxide radical anion O_2^-, hydrogen peroxide H_2O_2 and hydroxyl radical $OH^{\bullet}$ has been proposed to be involved in both inflammation and, at various stages, carcinogenesis.[1] Supporting evidence comes from the observation that their scavengers inhibit pathological processes.[2] Both metalloenzymes and small molecular weight transition metal complexes catalyse the dismutation of superoxide anions to oxygen and hydrogen peroxide as well as react promptly with hydrogen peroxide to give molecular oxygen and water.[3] Recent studies on copper(II) complexes with ligands of biological relevance have shown that indeed these species do behave as superoxide dismutase (SOD) analogues and evidence for copper protective action has come from many findings about pharmacological effects of certain copper complexes.[4–6] In order to have information on the catalytic activity of the copper(II) complexes an enzymatic assay has been employed. Two problems arose with this assay: (i) a very low concentration of the copper(II) complex needs to be used to follow their scavenging activity, (ii) at these micromolar concentrations the species distribution changes towards less co-ordinated species because water can be a competing ligand (therefore, sometimes, an excess of ligand has to be used in order to increase complex formation).[7–9] Moreover, since in pathological processes the concentration of reactive oxygen species can reach appreciable values, which overcome the human natural defences, the search for low molecular weight copper(II) complexes, which have a potential catalytic activity comparable to that of SOD, has continued in order to understand the mechanism by which these complexes exert their activity. Without a rational comprehension it is not possible to foresee applications for the use of these compounds as therapeutic or chemoprotective agents. Among the ligands which can co-ordinate copper, cyclopeptides have attracted particular interest owing to their constrained geometry. Cyclic peptides with specific amino acid residues such as histidine, glutamic and aspartic acids, methionine, tyrosine and lysine can be designed to have a close resemblance to the active site of metalloproteins.

In a preliminary paper[10] we reported the synthesis and a partial spectroscopic characterization of the histidine containing cyclopeptide cyclo(Gly-His)$_4$ which was designed as a potential analogue of the active site of SOD, because it provides four imidazole nitrogen atoms which might co-ordinate copper in a similar fashion. In subsequent papers, other workers[11,12] reported preliminary studies of the complexation features of the same cyclooctapeptide, and the linear tetrapeptide, H-(His-Gly)$_2$-OMe with Cu^{2+}, VO^{2+} and Mn^{2+} ions, but no evidence of the catalytic properties of these metal complexes against O_2^- was provided.

In this paper we report the synthesis of a novel cyclopeptide, cyclo(Gly-His-Gly)$_2$ (hereafter c-hexa), able to co-ordinate copper(II) ions through its imidazole or peptide nitrogen atoms. Moreover, the thermodynamic characterization of the proton and copper(II) complex species of cyclo(Gly-His)$_4$ (hereafter c-octa) and c-hexa as well as the spectroscopic characterization of copper(II) complexes are reported. A voltammetric study of the redox properties of these copper(II) complex species is also presented to give reasons for the antioxidant properties of selected complex species. A comparison with the recent findings reported in the literature[7–9,13–21] on the properties of analogous systems is made with the main goal to make clear the state of the art.

Table 1 Values of log β for proton and copper(II) complexes with cyclo(Gly-His)$_4$ and cyclo(Gly-His-Gly)$_2$ at 25 °C and $I = 0.1$ mol dm^{-3} (KNO_3) with estimated standard deviations in parentheses

Reaction	log β
c-octa + $H^+ \rightleftharpoons$ [Hc-octa]$^+$	7.03(1)
c-octa + 2 $H^+ \rightleftharpoons$ [H_2c-octa]$^{2+}$	13.57(2)
c-octa + 3 $H^+ \rightleftharpoons$ [H_3c-octa]$^{3+}$	19.61(3)
c-octa + 4 $H^+ \rightleftharpoons$ [H_4c-octa]$^{4+}$	25.14(3)
Cu^{2+} + c-octa + 2 $H^+ \rightleftharpoons$ [Cu(H_2c-octa)]$^{4+}$	18.87(1)
Cu^{2+} + c-octa + $H^+ \rightleftharpoons$ [Cu(Hc-octa)]$^{3+}$	14.375(4)
Cu^{2+} + c-octa $\rightleftharpoons$ [Cu(c-octa)]$^{2+}$	9.350(5)
c-hexa + $H^+ \rightleftharpoons$ [Hc-hexa]$^+$	6.79(1)
c-hexa + 2 $H^+ \rightleftharpoons$ [H_2c-hexa]$^{2+}$	12.79(1)
Cu^{2+} + c-hexa + $H^+ \rightleftharpoons$ [Cu(Hc-hexa)]$^{3+}$	10.42(1)
Cu^{2+} + c-hexa $\rightleftharpoons$ [Cu(c-hexa)]$^{2+}$	5.45(1)
Cu^{2+} + 2 c-hexa + $H^+ \rightleftharpoons$ [Cu{H(c-hexa)$_2$}]$^{3+}$	15.10(1)
Cu^{2+} + 2 c-hexa $\rightleftharpoons$ [Cu(c-hexa)$_2$]$^{2+}$	9.02(1)
Cu^{2+} + c-hexa $\rightleftharpoons$ [Cu(c-hexa)H_{-1}]$^+$ + H^+	−1.40(1)
Cu^{2+} + c-hexa $\rightleftharpoons$ [Cu(c-hexa)H_{-2}] + 2 H^+	−8.00(1)

Results and discussion

Thermodynamic results

Proton and copper(II) complexes. Protonation and copper(II) formation constants (log β) of c-hexa and c-octa are reported in Table 1. The literature data concerning protonation of histidine containing cyclopeptides show that, usually, the nitrogen basicity of the imidazole moieties in cyclopeptides[22,23] is lower than that of "free" imidazole (≈6.4 *vs.* 7.01).[24] In contrast, the log K of the first protonation step of c-octa (7.03) is very close to that of imidazole. Additionally, the log K of the *second* protonation step of c-octa (6.54) is very similar to those concerning the *first* step of histidine containing cyclopeptides (≈6.4). Finally, the log K of the *third* (6.04) and *fourth* (5.53) protonation steps are respectively, higher and comparable to that reported for the *second* step of cyclo-(L-His-L-His)[25] (cyhis) (5.49). This trend, which is quite anomalous because one should expect lower protonation step constants, could be explained taking into account that the larger c-octa ring might allow the four imidazole nitrogen atoms to behave quite independently of each other. However, we cannot exclude that also weak interactions (*e.g.* hydrogen bonds, "stacking" interactions, *etc.*) might contribute to the observed behaviour.

The first protonation constant of the smaller c-hexa (6.79) is just in between those of c-octa (7.03) and cyhis (6.53),[25] but its second protonation step constant is about 0.5 logarithmic units higher with respect to the analogous step of cyhis (6.00 *vs.* 5.49). Therefore, also in this case, it is conceivable to hypothesize that some "weak" interaction might play a role in this anomalous trend.

Both the stability and the stoichiometry of the complex species formed with copper(II) by c-hexa and c-octa reflect the different number of co-ordination sites available in the two ligands (see Table 1). For the Cu^{II}–c-octa system the formation of a precipitate hindered the pH-metric analysis above pH 6. "Best fit" pH-metric results were obtained by considering the following species: [Cu(H_2c-octa)]$^{4+}$, [Cu(Hc-octa)]$^{3+}$, [Cu(c-octa)]$^{2+}$. The species distribution diagram (Fig. 1) shows that the species which forms at higher percentage in the pH range 3.0–6.0 is [Cu(c-octa)]$^{2+}$. The stability constant for this species (9.35) is much higher than that reported for the 1:1 copper(II)–cyhis complex (6.01),[25] in which copper(II) is co-ordinated to two imidazole nitrogen atoms. Therefore, we can assume that more than two imidazole nitrogen atoms are involved in the co-ordination environment of the [Cu(c-octa)]$^{2+}$ species. However, this assignment, merely on the basis of the stability constant values, would be too speculative, whereas it can be reasonably done by means of visible optical and ESR data (see below).

For the copper(II)–c-hexa system the best fit was obtained considering the following species: [Cu(Hc-hexa)]$^{3+}$, [Cu(c-hexa)]$^{2+}$, [Cu{H(c-hexa)$_2$}]$^{3+}$, [Cu(c-hexa)$_2$]$^{2+}$, [Cu(c-hexa)H_{-1}]$^+$, [Cu(c-hexa)H_{-2}]. The major species in the pH range 3–7 are [Cu(Hc-hexa)]$^{3+}$, [Cu(c-hexa)]$^{2+}$ and [Cu(c-hexa)$_2$]$^{2+}$, whilst at higher pH the most abundant species is [Cu(c-hexa)H_{-2}] (Fig. 2). The presence of "only" two imidazole nitrogen atoms in c-hexa is readily reflected in the stability constant of the copper 1:1 complex which is similar to that of the analogous copper(II)–cyhis species.[25] On the other hand, it allows the formation of the [Cu(c-hexa)$_2$]$^{2+}$ complex, not observed in the analogous system with c-octa. It is reasonable to hypothesize that in the [Cu(c-hexa)$_2$]$^{2+}$ species copper(II) is co-ordinated to four imidazole nitrogen atoms, because the value of its formation constant is only slightly lower than that of [Cu(c-octa)]$^{2+}$.

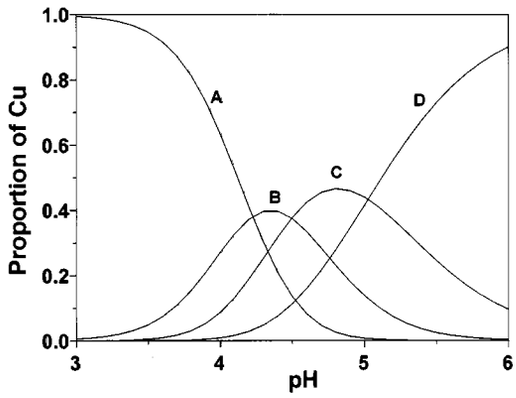


Fig. 1 Species distribution diagram for the copper(II)–c-octa system. [Cu]$^{2+}$ = 4 mmol dm^{-3}, [c-octa] = 8 mmol dm^{-3}. A = [Cu]$^{2+}$, B = [Cu(H_2c-octa)]$^{4+}$, C = [Cu(Hc-octa)]$^{3+}$, D = [Cu(c-octa)]$^{2+}$.

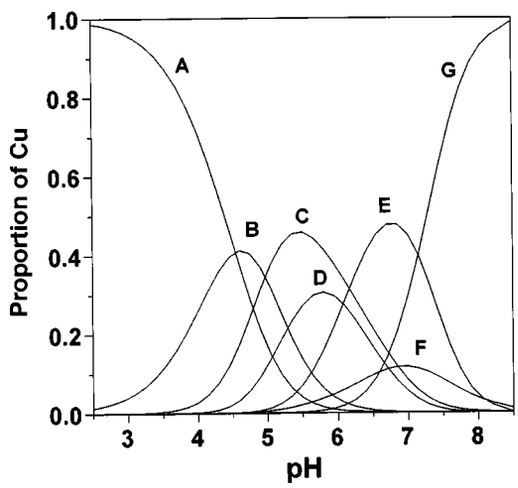


Fig. 2 Species distribution diagram for the copper(II)–c-hexa system. [Cu]$^{2+}$ = 4 mmol dm^{-3}, [c-hexa] = 8 mmol dm^{-3}. A = [Cu]$^{2+}$, B = [Cu(Hc-hexa)]$^{3+}$, C = [Cu(c-hexa)]$^{2+}$, D = [Cu{H(c-hexa)$_2$}]$^{3+}$, E = [Cu(c-hexa)$_2$]$^{2+}$, F = [Cu(c-hexa)H_{-1}]$^+$, G = [Cu(c-hexa)H_{-2}].

Spectroscopic results

The ESR spectra of copper(II) complexes with c-octa vary depending on the pH of the aqueous solution, thus suggesting that more than one complex species can be obtained, as already found in the pH-metric study. From a careful inspection of Table 2 it is possible to see there are at least three pH regions which are characterized by the presence of definite complex species. Unfortunately, because of precipitation problems during the pH-metric measurements, the distribution study was stopped at pH 6, in which the [Cu(c-octa)]$^{2+}$ species is predominant. However, after a certain while, on stirring the solution and slightly increasing its pH, the precipitates redissolve, thus allowing a spectroscopic study of other complex species. The shifts of the parallel magnetic parameters lead us to think

Table 2 ESR Frozen solution parallel spin Hamiltonian parameters, isotropic parameters and number of shf lines from room temperature ESR spectra, and visible optical data of copper(II) complexes with c-octa and c-hexa

pH	$g_{\parallel}$	$A_{\parallel}$/cm^{-1}	g_{iso}	A_{iso}/cm^{-1}	shf lines	λ/nm	ε/dm^3 mol^{-1} cm^{-1}	Ref.
Copper(II) complex species with c-octa								
5.5–6.5[a]	2.255(3)	0.0184(3)	2.134(5)	0.0082(4)	9	586	80	This work
7.0[b]	2.248(4)	0.0192(4)	2.136(5)	0.0092(4)	≈9	574	95	This work
7.5–9.5[c]	2.198(3)	0.0208(4)	2.109(5)	0.0093(4)	≈9	560	94	This work
Copper(II) complex species with c-hexa								
8.5[d]	2.223(4)	0.0171(5)	2.129(5)	0.0061(4)	≈9	624	130	This work
7.1[e]	2.256(3)	0.0186(3)	2.151(5)	0.0068(4)	9	618	57	This work
Copper(II)–imidazole complexes								
6.0[f]	2.253(2)	0.0190(2)	2.118(2)	0.0082(2)	9	—	—	25
7.0[g]	2.260(1)	0.0185(1)	2.131(3)	0.0079(3)	9	—	—	26
7.3[h]	2.247(2)	0.0190(2)	—	—	9	612	48	9
7.3[i]	2.246(2)	0.0189(2)	—	—	9	602	44	9
5.0[j]	2.254(2)	0.0184(2)	—	—	—	—	—	27

[a] $[Cu(c\text{-}octa)]^{2+}$. [b] $[Cu(c\text{-}octa)H_{-1}]^{+}$. [c] $[Cu(c\text{-}octa)H_{-2}]$. [d] $[Cu(c\text{-}hexa)H_{-2}]$. [e] Parameters pertaining to $[Cu(c\text{-}hexa)_2]^{2+}$ obtained from solutions in which the metal to ligand ratio was 1 : 20. [f] $[Cu(cyhis)_2]^{2+}$. [g] $[Cu(Him)_4]^{2+}$. [h] $[Cu(c\text{-}Asp\text{-}Asp\text{-}Hm_2)_2]^{2+}$ [c-Asp-Asp-Hm_2 = cyclo(-L-aspartyl-L-aspartyl-) bis(histamine)]. [i] $[Cu(c\text{-}Glu\text{-}Glu\text{-}Hm_2)_2]^{2+}$ [c-Glu-Glu-Hm_2 = cyclo(-L-glutamyl-L-glutamyl-) bis(histamine)]. [j] $[Cu(octaHG)]^{2+}$ (octaHG = H-His^1-Gly^2-His^3-Gly^4-His^5-Gly^6-His^7-Gly^8-OH).

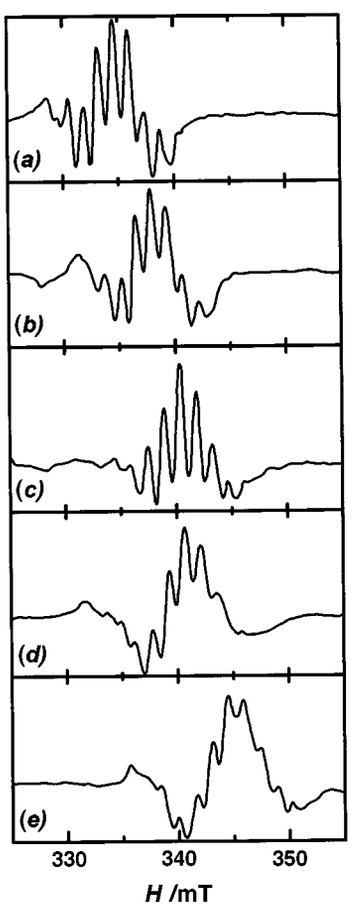
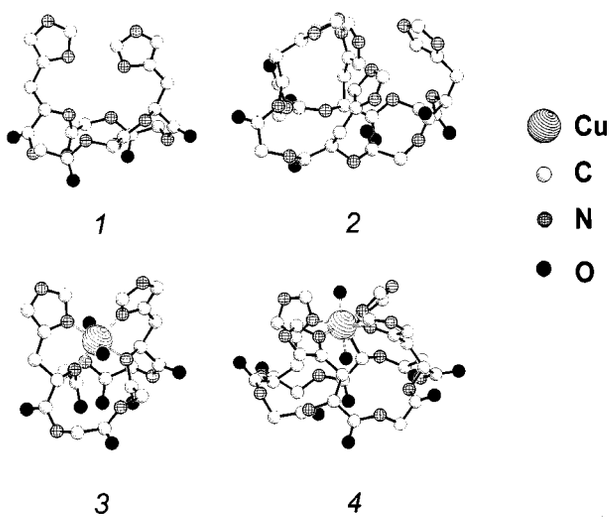


Fig. 3 Second derivative room-temperature ESR spectra of the highest field feature of copper(II) complexes with cyclopeptides (copper content 1 mmol dm^{-3}): (*a*) Cu–c-hexa system in ligand to metal ratio 20 : 1 at pH 7.1 ($A_{iso}^{N} = 1.4 \pm 0.1$ mT); (*b*) $[Cu(c\text{-}hexa)H_{-2}]$ at pH 8.5 ($A_{iso}^{N} = 1.4 \pm 0.1$ mT); (*c*) $[Cu(c\text{-}octa)]^{2+}$ at pH 6.0 ($A_{iso}^{N} = 1.5 \pm 0.1$ mT); (*d*) $[Cu(c\text{-}octa)H_{-1}]^{+}$ at pH 7.2 ($A_{iso}^{N} = 1.5 \pm 0.1$ mT); (*e*) $[Cu(c\text{-}octa)H_{-2}]$ at pH 9.0 ($A_{iso}^{N} = 1.5 \pm 0.1$ mT).

that two other species are important in the pH range 7.0–9.5, $[Cu(c\text{-}octa)H_{-1}]^{+}$ and $[Cu(c\text{-}octa)H_{-2}]$. All these species seem to be co-ordinated by four nitrogen donor atoms, as can be seen by the roughly resolved superhyperfine (shf) structure present in the room temperature spectra [see Fig. 3(*d*) and (*e*)]. In other words, the ESR parallel parameters obtained from the frozen solution spectra as well as the number of shf lines in the room-temperature spectra reflect the changes which occur in the copper(II) co-ordination environment on successive deprotonation of the peptide nitrogen atoms. At relative acid pH, the complex species $[Cu(c\text{-}octa)]^{2+}$ should only have donor imidazole nitrogen atoms, because they are the most easily deprotonable atoms [see Fig. 3(*c*), in which the 9-line pattern shf structure gives evidence of the four nitrogen co-ordination to copper]. Furthermore, this finding is confirmed by the similarity of the magnetic parameters associated with those pertinent to analogous $[Cu(Him)_4]^{2+}$ (Him = imidazole) systems, reported by the authors in previous studies.[9,25–27] Raising the pH and, thus, favouring the deprotonation of the peptide nitrogen atoms, $[Cu(c\text{-}octa)H_{-1}]^{+}$ and $[Cu(c\text{-}octa)H_{-2}]$ complex species form, as it can be seen from the shifts in their magnetic parameters ($g_{\parallel}$ values decrease and absolute values of parallel hyperfine coupling constants increase) as well as from blue shifts in their optical spectra (see Table 2). As suggested by their formula, one or two peptide nitrogen atoms substitute one or two imidazole nitrogen atoms in the co-ordination plane of the copper(II) complex. This fact is further confirmed by the comparison of their parallel Hamiltonian parameters with those pertinent to other four-nitrogen copper(II) complexes.[28,29]

In the case of the copper(II) complex with c-hexa, only the major species present at pH larger than 8 was examined, because at lower pH values there is large overlap among the complex species present in this system. The magnetic parallel parameters are different from those associated with the c-octa complex species existing in the same pH region. Moreover, also in this case, the room temperature ESR spectra revealed shf structure roughly ascribable to four nitrogen atoms [see Fig. 3(*b*)]. The c-hexa ligand contains two imidazole nitrogen atoms and several peptide nitrogen atoms which can undergo deprotonation on raising the pH of the system. Perhaps this ligand does not possess the same flexibility as the c-octa ligand, so the formation of a deprotonated complex species by means of two peptide and two imidazole nitrogen atoms constrains the copper(II) complex geometry to be distorted. All these geometrical factors are reflected in its parallel magnetic parameters, which show higher $g_{\parallel}$ and lower $A_{\parallel}$ with respect to the analogous complex species of c-octa at pH 7.5–9.5, as would have been expected for in plane four-nitrogen co-ordination distorted towards a tetrahedral situation. The red shift in its optical spectra and a higher molar absorption coefficient (see Table 2) than that of the analogous complex with c-octa confirm the above conclusions.

Computations carried out with the aid of the HyperChem software package[30] (copper atoms were linked to imidazole or peptide nitrogen atoms of c-hexa or c-octa and to two oxygen atoms of water molecules *in vacuo* and the energy of the resulting system was minimized; the calculation was performed by the MM^+ force field using the standard parameters of the package, the minimization using Fletcher–Reeves' conjugate gradient method and stopped when the root-mean-square gradient reached a value of 0.1 kcal $Å^{-1}$ mol^{-1}) on these copper(II) complexes supported the conclusions drawn on the basis of the spectroscopic data, even if these computed models have to be taken with great care when metal ions are present. Models of the two cyclic ligands, c-hexa and c-octa (structures 1 and 2, respectively) and of [Cu(c-hexa)H_{-2}] and $[Cu(c\text{-}octa)]^{2+}$ complexes (structures 3 and 4, respectively) are shown below.[25] In both complexes copper(II) shows its usual apically elongated octahedral geometry formed by means of four nitrogen donor atoms in the equatorial plane and two water molecules linked in the apical positions. Of interest is the formation of macrochelate rings around copper. The geometry of the [Cu(c-octa)H_{-2}] complex (not shown) is analogous to that of [Cu(c-hexa)H_{-2}], but a little more regular.



When an excess of c-hexa ligand was added to the solution containing the copper(II)–c-hexa system ($[Cu^{2+}]$ = [c-hexa] = 1 mmol dm^{-3}) at pH ≈7 a bis $[Cu(c\text{-}hexa)_2]^{2+}$ species can be detected. It is possible to state on the grounds of the similarity of the magnetic parameters with those of $[Cu(c\text{-}octa)]^{2+}$ and of analogous Cu–imidazole systems[9,25–27] that, in both cases, four imidazole nitrogen atoms are engaged in the co-ordination to copper (see Table 2).

Voltammetric results

Cyclic voltammograms run on the solutions of these complexes are characteristic of quasi-reversible one-electron redox processes, because large $E_{pc} - E_{pa}$ values were found [Fig. 4 shows the cyclic voltammogram on an aqueous solution containing the copper(II)–c-hexa system at pH 8.5]. Successive scans did not alter the voltammetric pattern and, therefore, disproportionation reactions as a consequence of the reduction of copper(II) to copper(I) can be excluded. The $E°$ values were reported in Table 3. This behaviour is probably to be ascribed to the oxidation of a copper(I) species having a different stereochemistry from that of the original copper(II) complex. In fact, copper(I) complexes usually prefer trigonal planar, tetrahedral or linear stereochemistries,[33] whilst copper(II) complexes usually have tetragonally elongated pseudo-octahedral geometries. During the reduction process a stereochemical rearrangement could occur in order to meet the co-ordination requirements of the reduced copper(I) species. These stereochemical rearrangements can be tetrahedral distortions of the in plane bound donor atoms or effective partial leaving of the ligands from the co-ordination sphere. Anyway, since the voltammetric behaviour does not change in successive scans, it is possible to assume that these stereochemical changes are reversible and do not alter the identity of the original copper(II) complex under-

Table 3 The IC_{50} values taken from recent reports on SOD-like activities of copper(II) complexes. Known redox potentials for mononuclear (one-electron transfer) and for dinuclear copper(II) complexes (two-electron transfer) have been added for comparision, charges are omitted for the sake of simplicity

Complex	$E°$/mV	10^8 IC_{50}/mol dm^{-3}	Ref.
BESOD	320	1 ÷ 4	7, 8, 31
[$Cu(CDhm_2^{AB})$]		12	20
[Cu(Mc)]		13	18
[Cu(TAAB)]	155	14	32
[$Cu(CDhm_2^{AC})$]		18	20
[$Cu(c\text{-}Asp\text{-}Asp\text{-}Hm_2)_2$]	215	25	9
[$Cu(c\text{-}Glu\text{-}Glu\text{-}Hm_2)_2$]	333	25	9
[$Cu_2(bpzbiap)Cl_3$]	153	26	21
[$Cu_2(EGTB)(bipy)Cl_4$]	86	35	16[a]
[$Cu(CDhm_2^{AD})$]		30	20
[$Cu(PheA)_2H_{-2}$]	18	43	17
[$Cu(c\text{-}His\text{-}His)_2$]		50	7
[Cu(im)Cu(Mbc)]	−26	50	19[b]
[$Cu(ValA)_2H_{-2}$]	−36	56	17
[Cu(c-octa)]	269	80	This work
[$Cu(ProA)_2H_{-2}$]	−52	82	17
[$Cu(Leu\text{-}Leu)H_{-1}$]	−61	130	7, 17[c]
[$Cu(c\text{-}octa)H_{-2}$]	195	140	This work
[$Cu(c\text{-}hexa)H_{-2}$]	219	160	This work
[$Cu(GHGH)H_{-2}$]		160	15
[$Cu(GHL)H_{-1}$]	−132	532	7, 17

Abbreviations: $CDhm_2^{AX}$ = β-cyclodextrins 6A-6X difunctionalized with histamine (imidazole-4-ethanamine), Mc = macrocyclic ligand formed by 1,3-bis(5-methylpyrazol-1-yl)propane and 1,3-bis(diethylamino)propane; TAAB = tetraanhydroaminobenzaldehyde; c-Asp-Asp-Hm_2 = cyclo(-L-aspartyl-L-aspartyl-) bis(histamine); c-Glu-Glu-Hm_2 = cyclo(-L-glutamyl-L-glutamyl-) bis(histamine); bpzbiap = 3-[bis(imidazol-2-yl)methyl]-1,5-bis(pyrazol-1-yl)-3-azapentane; EGTB = *N,N,N′,N′*-tetrakis(benzimidazol-2-ylmethyl)-1,4-diethylene amino glycol ether; PheA = L-phenylanalinamide; ValA = L-valinamide; ProA = L-prolinamide; Mbc = macrobicyclic ligand. [a] Among the dinuclear complexes reported by the authors, this showed a reasonable activity with a quasi-reversible two-electron reduction. [b] Not in water, but dimethylamide solution; $E° = \frac{1}{2}(E_{p_c} + E_{p_a})$. [c] Redox potential extrapolated from data on the analogous complexes with L-alanyl-L-alanine and L-alanyl-L-leucine.

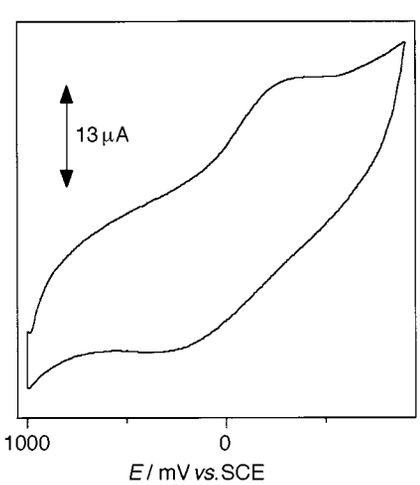


Fig. 4 Cyclic voltammogram associated with the copper reduction and oxidation from 1 mmol dm^{-3} [Cu(c-hexa)H_{-2}] solution recorded at a carbon electrode at pH 8.5. Electrolyte 0.1 mol dm^{-3} KNO_3, potential sweep rate 200 mV s^{-1}.

going reduction. The most easily reducible copper(II) complex was $[Cu(c\text{-}octa)]^{2+}$ (E° = 269 mV). As seen [34] for other copper(II) complexes, the presence of peptide nitrogen atoms in the co-ordination sphere "does not help" the reduction to copper(I) species for at least two reasons: (i) the complex equatorial field strengthens, and, therefore the copper(II) complex is stabilized, (ii) the co-ordinated peptide nitrogen atoms impose a more rigid situation, which is not favourable to the formation of typical copper(I) complex stereochemistries. In fact, between $[Cu(c\text{-}hexa)H_{-2}]$ and $[Cu(c\text{-}octa)H_{-2}]$ which are species with the same co-ordination level with two imidazole and two peptide nitrogen atoms, there is only a little difference (219 against 195 mV, respectively). It is interesting that these redox behaviours, in a first approximation, parallel the ability of these complexes to behave as SOD analogues as we will show later [see E° values reported in Table 3 together with those pertaining to other copper(II) complexes, the SOD-like activity of which was previously tested].

Superoxide dismutase-like activity

As we said in the introduction the knowledge of the correct speciation of the system under the experimental condition of the enzymatic assay plays a fundamental role in determining any reasonable catalytic activity by these complexes. Hence, we made an enzymatic assay by using an excess of ligand, in particular, a ligand to metal ratio of 5:1 and micromolar amounts of copper. Under these conditions at two pH values 6.2 and 7.8, the simulation procedure of the species distribution (see Experimental section) roughly gave 62% of the $[Cu(c\text{-}octa)]^{2+}$, 72% of $[Cu(c\text{-}octa)H_{-2}]$ and 71% of $[Cu(c\text{-}hexa)H_{-2}]$ species. In the case of $[Cu(c\text{-}octa)H_{-2}]$, since we were not able to determine any formation constant for the deprotonated species, because of precipitation problems, to have an idea of the amount of formed species we assumed that the deprotonation of two peptide nitrogen atoms in the copper(II)–c-octa system gave rise to a similar contribution to that found for the copper(II)–c-hexa system. Once it was verified that the free cyclic peptides do not contribute to the catalytic activity as well as free copper(II) ions, copper(II) xanthine and copper(II) phosphate species which are practically absent as significant species, an IC_{50} of 0.8 μmol dm^{-3} for the $[Cu(c\text{-}octa)]^{2+}$ species at pH 6.2 and 1.4 μmol dm^{-3} for $[Cu(c\text{-}octa)H_{-2}]$, and 1.6 μmol dm^{-3} for $[Cu(c\text{-}hexa)H_{-2}]$ at pH 7.8 were found. It is worth noting that the best catalytic activity is shown by $[Cu(c\text{-}octa)]^{2+}$ whereas $[Cu(c\text{-}octa)H_{-2}]$ and $[Cu(c\text{-}hexa)H_{-2}]$ give rise roughly to the same value of IC_{50} following the trend shown by their redox potentials (see Table 3). A four imidazole nitrogen co-ordination environment in a flexible arrangement may favour the catalytic activity against O_2^- as both Cu^{II} and Cu^{I} can be accommodated in a proper co-ordination geometry, so that the reduction potential would be in a good range (from +980 to −450 mV) for interacting with O_2^-.[35]

Conclusion

Copper(II) complexes with c-octa and c-hexa easily form in aqueous solutions giving rise to two definite complex species in the pH region around 6 and above 8, $[Cu(c\text{-}octa)]^{2+}$ and $[Cu(c\text{-}octa)H_{-2}]$, and above 8, $[Cu(c\text{-}hexa)H_{-2}]$, respectively. Our thermodynamic and spectroscopic study has shown that a four-nitrogen co-ordination environment around copper is achieved by means of imidazole or imidazole and peptide nitrogen atoms, depending on the pH of the aqueous solution. Furthermore, these complex species possess peculiar redox behaviour as well as scavenging properties against O_2^-. It is quite interesting to have found out that, at the same co-ordination level, the redox properties of these compounds parallel their scavenging abilities against O_2^- and that the easily reducible complex is the more flexible $[Cu(c\text{-}octa)]^{2+}$ because of the co-ordination of the side-chain imidazole nitrogen atoms of the cyclooctapeptide. Unfortunately, their scavenging ability is not comparable with that of other systems, which tend to the limit of SOD, in spite of their highly positive redox potentials. Table 3 reports a list of copper(II) complexes which have been shown to behave as SOD analogues and hence as catalysts for the O_2^- dismutation reaction. The IC_{50} values as well as redox potentials (where known) were taken from the recent literature reports, together with the analogous values obtained in the case of bovine erythrocyte superoxide dismutase (BESOD).[31] As shown in Fig. 5 there is no linear correlation between E° values (calculated in the case of quasi reversible processes too) and IC_{50} values obtained from the enzymatic assays, unless we restrict the redox potentials in the range from −100 to 350 mV or to an homologous series of complexes. This pattern could be probably due to the following reasons.

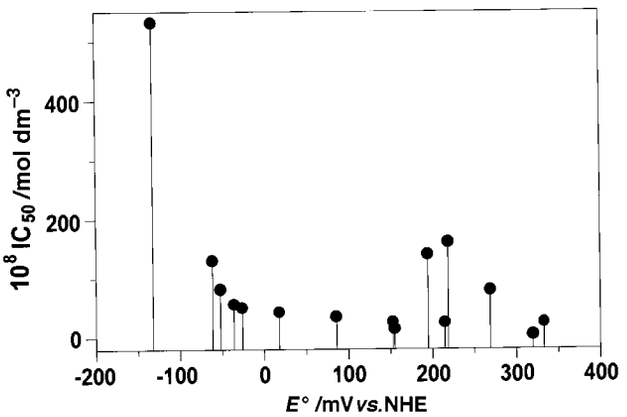


Fig. 5 Plot of the catalytic activity against O_2^- (IC_{50}) as a function of redox potentials of the copper(II) complexes reported in Table 3.

(1) The catalytic activity is not due to a redox reaction only, but needs the formation of labile adducts, as well as the involvement of molecular recognition processes, which are different things from a single electron transfer process. It can be stated that the relationship between the increase of the redox potential and the catalytic activity works well within an homologous series of complexes.

(2) A flexible co-ordination polyhedron together with the presence of accessible sites are pre-requisites [they make easier the reduction to copper(I) species] for an acceptable SOD-like activity. As shown by model structures of the copper(II) complexes reported above, in consequence of the co-ordination (especially when peptide nitrogen atoms are involved) the metal site is buried in the ligand cavity.

All the molecular recognition processes (*i.e.* occurrence of weak interactions) which can drive O_2^- towards the metal catalytic site should play a favourable role. In this perspective, it is important to note how the presence of a β-cyclodextrin cavity to which a bidentate ligand like histamine is covalently attached to two different glucose rings could mimic weak interactions present in metalloproteins.[20]

Noteworthy, the formation of the $[Cu(c\text{-}octa)]^{2+}$ and $[Cu(c\text{-}hexa)]^{2+}$ species implies the formation of macrochelate rings, suggesting a certain preorganization of the ligands, as underlined by the anomalous protonation constant values.

Experimental

Synthesis of cyclo(Gly-His-Gly)$_2$

This cyclopeptide was obtained by cyclization of the linear precursor Gly-His-Gly-Gly-His-Gly which was synthesized on a Milligen/Biosearch 9050 Peptide Synthesizer using *N*-fluoren-9-ylmethoxycarbonyl (Fmoc) amino acid pentafluorophenyl esters. The Fmoc-His(*N*-im Boc) pentafluorophenyl ester was used for the coupling of the histidyl residue (Boc = *tert*-

butoxycarbonyl). The peptide was assembled starting from Fmoc-Gly-KA resin. The linear peptide was cleaved from the resin with 95% trifluoroacetic acid in water. The product was purified on a CM Sephadex C-25 (NH_4^+ form) column and characterized by means of FAB-MS ($[M + H]^+$, *m/z* 521). The cyclization of the linear hexapeptide was carried out in dry dmf under high dilution using the BOP/DIEA method[36,37] [BOP = benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate, DIEA = diisopropylethylamine]. The reaction mixture was allowed to react for 1 d. In order to reduce undesired side reactions such as cyclodimerization or polymerization, the open-chain precursor was slowly added over a period of 12 h using a syringe pump infuser. The cyclo(Gly-His-Gly)$_2$ was purified on a CM Sephadex C-25 (NH_4^+ form) column and eluted with a linear gradient from 0 to 0.15 mol dm^{-3} of aqueous NH_4HCO_3. The pure cyclic peptide showed a negative ninhydrin test and a positive Pauly test[38] indicating the absence of free terminal NH_2 and the presence of unsubstituted histidine side chains, respectively. High performance liquid chromatography (HPLC) analysis of the cyclic peptide on Spherisorb ODS-2 (5 mm) in a 125 × 4 mm column showed a single peak [eluent: (A) water with 0.1% CF_3CO_2H; (B) CH_3CN with 0.1% CF_3CO_2H; linear gradient, 0 to 30% (B) in 30 min; flow rate 1 ml min^{-1}; detection 220 nm]. The FAB-MS spectrum showed a molecular peak at $[M + H]^+$, *m/z* 503; no other peaks were detected at higher molecular weights. Proton NMR spectra were obtained at room temperature in D_2O on a Bruker ARX-250 spectrometer, operating at 250 MHz, in the presence of a stoichiometric amount of CF_3CO_2D, due to the limited solubility of the cyclopeptide in water. The spectrum is very simple and shows signals consistent with the cyclic structure: δ 3.28 (ddd, 4 H, His β-CH_2); 3.98 (m, 8 H, Gly α-CH_2), 4.65 (dd, 2 H, His α-CH); 7.34 (s, 2 H, His CH-4) and 8.65 (s, 2 H, His CH-2).

Synthesis of cyclo(Gly-His)$_4$

The synthesis and characterization of the cyclopeptide cyclo(Gly-His)$_4$ has been made by following the above-mentioned procedure and was previously reported.[10]

Electromotive force measurements

Fully automated sets of Metrohm burette, meter, and combined electrode (E665, E654 and EA125), computer controlled, were used to have data on proton and copper(II) complex formation. All experiments at 25 ± 0.1 °C were carried out in thermostatted cells in which the solutions at I = 100 mmol dm^{-3} KNO_3 were magnetically stirred and maintained under an inert nitrogen atmosphere. The solutions containing the ligands and the copper(II) were titrated with CO_2-free KOH. The concentrations of both cyclopeptides were kept in the range 4–8 mmol dm^{-3} and the copper(II) to ligand ratio was in the range from 1:1 to 1:2. Other details were as previously reported.[9,15] The ACBA,[39] SUPERQUAD[40] and DISDI[41] computer programs were employed to refine acid–base titration parameters, obtain reliable data on formation constants and determine the species distribution, respectively.

Spectroscopic measurements

Frozen solution ESR spectra were recorded on a Bruker ER 200 D spectrometer equipped with the 3220 data system at 150 K. The 1 mmol dm^{-3} copper(II) complex solutions were prepared *in situ* by mixing the necessary volume of a standard solution of $^{63}Cu(NO_3)_2$ with an equimolar solution of the pertinent ligand and adjusting the pH of the resulting solution by adding 10 mmol dm^{-3} KOH or HNO_3. Methanol or glycerol not exceeding 10% was added to the aqueous copper(II) complex solutions to increase resolution (usually this addition doesn't substantially influence the species distribution). Room temperature ESR spectra were obtained by using a Bruker quartz aqueous solution flat cell. Second derivative spectra of the high field copper 4th line have been recorded to give evidence of shf structures present in the experimental room temperature spectra. Frozen solution spectra did not reveal overlap of signals coming from the various species which could be present in these systems because the pH of the aqueous solution was chosen so as to maximize the percentage of formation of the selected species. Parallel spin Hamiltonian parameters from frozen solution spectra have been obtained directly from the experimental spectra, recorded on an enlarged scale, calculated from the 2nd and 3rd lines in order to get rid of second order effects.[42] Moreover, to have an idea of the possible geometries of these complexes, geometry optimization has been carried out by making use of HyperChem[30], and the results compared with the shifts observed in the magnetic parameters obtained for each complex species.

Visible optical spectra were obtained with a diode-array Hewlett-Packard 8452A spectrophotometer on 1 mmol dm^{-3} copper(II) aqueous solutions contained in quartz cells.

Voltammetric measurements

Cyclic voltammograms have been obtained with an Amel 473 analyzer equipped with an Amel 863 recorder. The 1 mmol dm^{-3} copper(II) solutions at different pH values have been analysed at 25 ± 0.1 °C using a glassy carbon electrode as working electrode (3 mm diameter), platinum and saturated calomel electrodes as counter and reference electrodes, respectively. All the measurements were performed in a 5 ml Amel glass cell in which 100 mmol dm^{-3} KNO_3 was used as supporting electrolyte and in accordance with the complex formation study and the ESR results the solution pH was set at the value at which a definite species is present. Copper(II) complex solutions were degassed by using ultra-pure nitrogen, which was previously passed over copper wires at 400 °C. The electrode processes are to be considered quasi-reversible ($k_s = 3 \times 10^{-4}$ cm s^{-1}),[43] and thus the peak potentials are dependent both on the concentration of the complex and on the potential sweep rate. To analyse these voltammograms, the ESP program (electrochemical simulation package, version 2.3) was used,[44] assuming that the redox behaviour could be accounted for by an electrochemical step-chemical step mechanism.[45] The $E°$ values thus calculated were referred to the NHE, taking into account that the SCE(water) $E°$ *vs.* NHE(water) is +0.244.[46]

Superoxide dismutase activity measurements

Superoxide-like activity was determined as reported in previous papers,[8,14,17,18] by enzymatically (xanthine–xanthine oxidase system) generating O_2^- and following the reduction of cytochrome c at 550 nm, in 10 mmol dm^{-3} phosphate buffered solutions at two different pH values, 6.2 and 8, and using a concentration of copper(II) complex equal to or lower than 1 μmol dm^{-3}. All measurements were carried out at 25 ± 0.1 °C using 1 × 1 cm thermostatted cuvettes, in which solutions were magnetically stirred. Bovine SOD was also monitored with this system in order to make a comparison of the results on the O_2^- dismutation ability by these copper(II) complexes with the literature results on similar systems.

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