Copper(II) Binding Modes in the Prion Octapeptide PHGGGWGQ: A Spectroscopic and Voltammetric Study

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Abstract: The N-terminal octapeptide repeat region of human prion protein (PrP^c) is known to bind Cu^{II}. To investigate the binding modes of copper in PrP^c, an octapeptide Ac-PHGGGWGQ-NH_2 (**1**), which corresponds to an octarepeat sequence, and a tetrapeptide Ac-HGGG-NH_2 (**2**) have been synthesised. The copper(II) complexes formed with **1** and **2** have been studied by circular dichroism (CD) and electron spin resonance (ESR) spectroscopy. Both peptides form 1:1 complexes with Cu^{II} at neutral and basic pH. CD, ESR and visible absorption spectra suggest a similar co-ordination sphere of the metal ion in both peptides, which at neutral pH consists of a square pyramidal geometry with three peptidic nitrogens and the imidazole nitrogen as donor atoms. Cyclic voltammetric measurements were used to confirm the geometrical features of these copper(II) complexes: the observation of negative redox potentials are in good agreement with the inferred geometry. All these results taken together suggest that peptide **1** provides a single metal binding site to which copper(II) binds strongly at neutral and basic pH and that the binding of the metal induces the formation of a stiffened structure in the HGGG peptide fragment.

Keywords: circular dichroism · copper · cyclic voltammetry · EPR spectroscopy · peptides · prion

Introduction

Metal complexes with peptide ligands are widely studied because they can be considered a simplified, but representative, model of biological metal–protein interactions. During the past decade we have studied several copper(II) complexes with linear or cyclic peptides.[1–7] Such complexes were thermodynamically and spectroscopically characterised and their capability to act as scavenger of O_2^- tested.[3, 5, 6] From these studies it has been possible to obtain useful information about the mechanism by which these complexes exert their antioxidant activity and then address the relationship between the measured activity and their structural properties.[3, 5, 6]

Another useful strategy to study metal–peptide interactions employs metal complexation to achieve the folding of polypeptidic chains into well-defined conformations.[8] The increasing number of reports about the introduction of metal binding sites into native and/or synthetic proteins,[9] as well as appropriately designed oligopeptides,[7, 10] reveal that this is a currently active research field, which can provide useful information for the development of synthetic metalloproteins. These models can be tailored for specific biological functions or to exploit the role played by metal ions in the folding of proteins. This latter aspect has a remarkable importance because there is mounting evidence that suggests a direct pathogenic role for several transition metals in neurodegenerative disorders related to defective protein folding, including Alzheimer's and prion diseases.[11] In particular, in prion diseases the critical pathogenic event is the misfolding of the prion protein (PrP^c), which is expressed in the brain, to form an abnormal pathogenic isoform (PrP^{Sc}) believed to cause a series of neurodegenerative diseases that include Creutzfeldt-Jakob disease (CJD) in humans and bovine spongiform encephalopathy (BSE) in animals.[12] Despite intensive biochemical and biophysical studies, the molecular mechanism which results in the conformational transition from PrP^c to PrP^{Sc} and the subsequent neurodegeneration remains unknown. The recent findings that indicate that PrP^c can exist in vivo as the Cu-metalloprotein form[13] raise the possibility that the prion protein is involved in copper metabolism in the central nervous system.[13, 14] However, early studies revealed that cuprizone, a copper chelating

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agent, induces neuropathological changes in mice similar to those found in prion diseases;[15] this suggests a role for copper in the pathophysiology of prion disease. So on one hand, PrP^c may regulate the Cu^{II} homeostasis by acting as a shuttle for copper ions destined to bind to enzymes that prevent oxidative damage such as superoxide dismutase.[13–14] On the other hand, copper binding, or rather the lack of it, may play a crucial role in the pathogenesis of prion diseases.[16] Therefore, it is important to investigate the mode and the site of binding of copper(II) to the prion protein. Prions selectively bind copper in their N-terminal domain, which contains a group of octarepeats with the consensus sequence of Pro-His-Gly-Gly-Gly-Trp-Gly-Gln.[13a, 17] Several studies have aimed to elucidate the structural nature of the copper complex by using short peptides that correspond to the octapeptide repeat motif and also the whole protein.[13b, 17, 18] However, the nature of the binding site as well as the number of metal ions involved is still a matter of debate.[13, 18]

In this study, we have examined the circular dichroism (CD), absorption and electron spin resonance (ESR) spectra of the copper(II) complex species formed by the peptides Ac-Pro-His-Gly-Gly-Gly-Trp-Gly-Gln-NH_2 (PHGGGWGQ; **1**) and Ac-His-Gly-Gly-Gly-NH_2 (HGGG; **2**), which are homologous with one of the octarepeats and a part of it, respectively. In addition, voltammetric measurements carried out on such copper(II)-octapeptide and -tetrapeptide complexes allowed us to get further insight into the coordination environment and redox properties of the metal site.

Results and Discussion

Circular dichroism (CD): Figure 1 shows the CD spectra of **1** recorded in the absence of copper(II) in water at different pH values. The main features present in these spectra are an intense minimum at about 200 nm and a positive band with a maximum around 224 nm. A change in intensity is observed between pH 6 and 7 in addition to a slight shift of the minimum towards shorter wavelengths. The CD spectrum of **1** is indicative of unordered chain conformation.[19] However

Abstract in Italian: *E' noto che la regione N-terminale della proteina umana del prione (PrP^c) contiene una regione in cui si ripete una sequenza ottapeptidica capace di legare il rame(II). Sono stati sintetizzati l'ottapeptide Ac-PHGGGWGQ-NH_2 (**1**), ed il tetrapeptide Ac-HGGG-NH_2 (**2**). I complessi di rame(II), studiati con tecniche spettroscopiche (CD ed ESR) in soluzioni acquose neutre, hanno geometrie piramidali a base quadrata, in cui la sfera di coordinazione del metallo contiene una molecola d'acqua e tre azoti in piano ed un'azoto apicale. In soluzione basica si ha un'espansione della coordinazione con formazione di geometrie pseudo-ottaedriche. Studi voltammetrici hanno rivelato potenziali redox negativi per queste specie, confermando le ipotesi strutturali ricavate dai dati spettroscopici. Questi risultati mostrano che questi peptidi contengono un sito preorganizzato per il rame e che in conseguenza della coordinazione essi assumono forme particolarmente strutturate.*

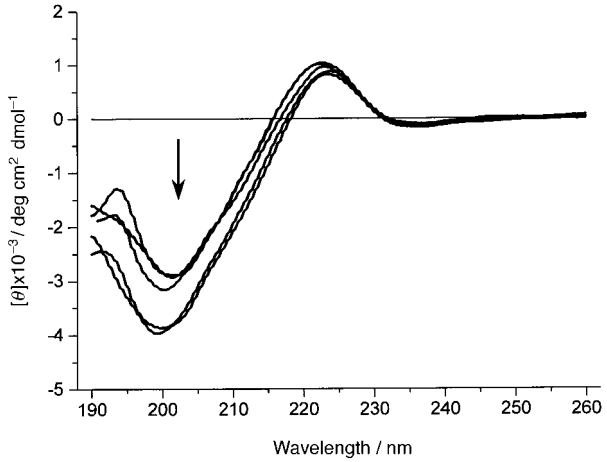

Figure 1. CD spectra of **1** ($c = 2.3 \times 10^{-4}$ M) in H_2O at different pH values in the absence of copper(II). From top to bottom (arrow): pH 4.0, 5.1, 6.0, 7.2, 8. 0.

the pH dependence shows that protonation of the histidine residue may have a weak effect on the conformation of the octapeptide **1** as evidenced by the shift of the minimum towards longer wavelengths at pH 4.

Copper(II) complexation affects the secondary structure of **1**; this is clearly shown by the CD spectra collected at various pH values (Figure 2). The pH titration was carried out in the

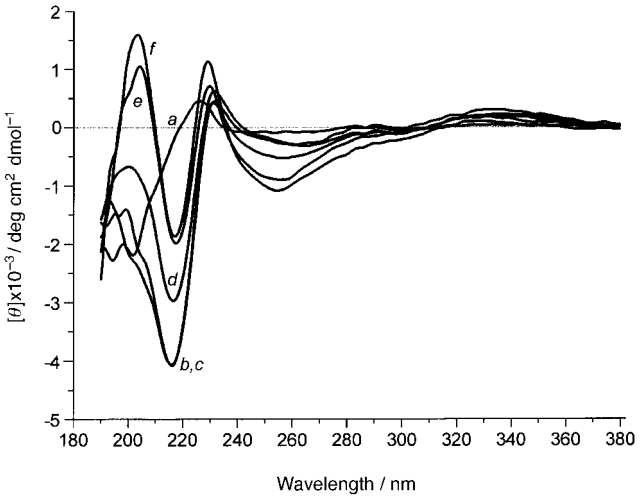

Figure 2. CD spectra of the copper(II)-**1** complex ($c = 2.3 \times 10^{-4}$ M) in H_2O at different pH values. a) pH 6.0; b) pH 7.3; c) pH 7.8; d) pH 8.9; e) pH 9.6; f) pH 10. 0.

range of pH 3 – 10 and in the presence of one mole-equivalent of $CuSO_4$. Below pH 6 the CD spectra did not show significant differences when compared to those of the free ligand at the same pH values. This pH dependence, indicates that the copper(II) ion binds tightly to **1** at pH > 6 and suggests an involvement of the histidine residue in the coordination of the metal ion. The CD spectrum obtained at pH 7.3 is characterised by a decrease in the negative ellipticity at 200 nm, a strong negative signal at 216 nm and a weaker positive maximum at 231 nm. The same trend is retained at pH 8, but on increasing the pH further, the negative ellipticity at 216 nm decreases and the growth of a positive signal at

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about 203 nm is observed. All these spectral changes are indicative of structure formation but rather than being consistent with any known CD spectrum of the common α-helix or β-sheet secondary structures, they are somewhat reminiscent of turn motifs. However, it should be said that in all the spectra recorded in the presence of copper(II), an unusual positive signal at approximately 230 nm is observed. This signal, which may come from the shift of the positive signal at 224 nm observed in the uncomplexed peptide, has been assigned to an aromatic transition of the tryptophan side chain[20]. Additional CD signals are observed at 254 nm and over 300 nm and have been assigned to optically active ligand-to-metal charge-transfer transitions (LMCT) that may occur from imidazole or deprotonated peptide nitrogens to the copper ion.[21]

The copper(II) binding properties of the peptide HGGG **2**, whose sequence is contained in octapeptide **1**, were also explored. In Figure 3, the CD spectra of the uncomplexed

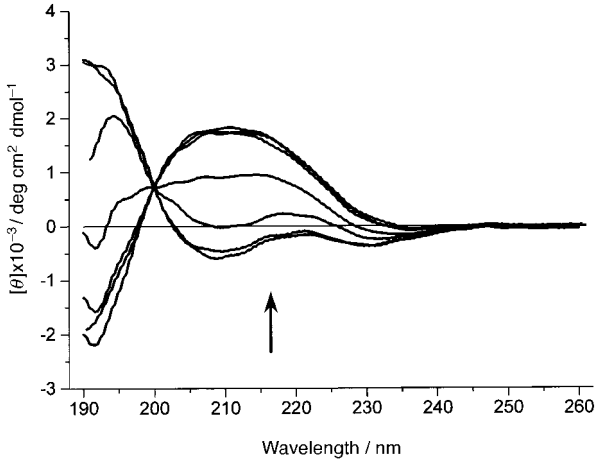

Figure 3. CD spectra of **2** ($c = 4.0 \times 10^{-4}$ M) recorded in H_2O at different pH values in the absence of copper(II). From bottom to top (arrow): pH 3.2, 4.2, 5.1, 6.1, 7.4, 8.0, 9.0.

peptide **2** taken in water at different pH values are shown. Helix-like CD spectra with low intensity are observed at pH 3.2 and 4.2. For small peptides, such class C spectra are frequently associated with the presence of β-turn conformations.[19] Varying the pH between 5.1 and 7.4 results in a modification of the CD pattern. At pH 7.4, the CD spectrum is characterised by a broad positive band between 200 and 230 nm and by negative ellipticity below 195 nm. These spectral features, which remain virtually unchanged up to pH 9, are less easily interpreted and cannot be compared with the known CD spectra for reverse turns. We can tentatively suggest that at acidic pH values the protonated imidazole side chain of histidine may form an intra-chain hydrogen bond that allows peptide **2** to adopt a folded conformation with substantial β-turn character. At higher pH values, the stabilising contribution of the imidazole side chain is lowered since deprotonation occurs. Therefore, the resulting CD spectra may reflect the presence of conformational mixtures in which random conformers begin to intrude. In this respect the observation of an isodichroic point at around 200 nm (see Figure 3), which suggests an equilibrium between two conformational states, is in agreement with this hypothesis.

The pH titration in the presence of copper(II) was carried out under the same experimental conditions used for peptide **1** and is reported in Figure 4. The CD spectra of the copper(II)

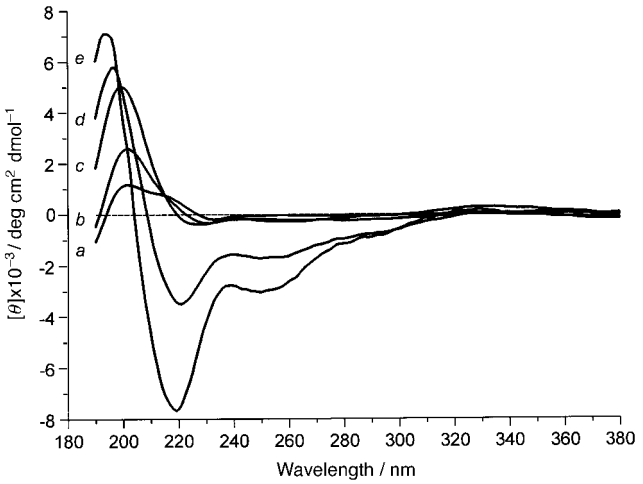

Figure 4. CD spectra of copper(II)-**2** complex ($c = 3.5 \times 10^{-4}$ M) in H_2O at different pH values: a) pH 6.1; b) pH 7.2; c) pH 8.0; d) pH 9.1; e) pH 10.0.

-**2** complex collected at pH < 6 did not show substantial differences in comparison with those obtained for the free peptide ligand at the same pH values. In this respect it behaves like peptide **1**.

The CD spectra a, b, and c recorded in the range of pH 6–8 appear to be related (Figure 4). The most common, and distinct, spectral type associated with a type II β-turn, classified as class B spectrum, is characterised by negative ellipticities > 220 nm and < 190 nm and a positive ellipticity in the 200–210 nm range.[19] All the spectra a, b and c of Figure 4 show minima and maxima almost in the appropriate wavelength range for a type II β-turn. Dramatic changes, in terms of signal intensities, are observed at higher pH values (Figure 4, curves d and e). In particular, as the pH is raised from 9 to 10, an increase and blue shift of the positive signal and the growth of two negative bands near 220 nm and 250 nm are observed. The CD spectrum of the copper(II) complex with peptide **2** obtained at pH 10 is characterised by a maximum at 195 nm along with negative ellipticity at 219 nm and 254 nm. If we consider the spectral region below 240 nm, this CD spectrum still resembles that of a type II β-turn,[19] but here both the maximum and minimum are slightly shifted to shorter wavelengths. In analogy to peptide **1**, the negative signal at 254 nm may be identified as a charge-transfer transition.

CD spectra of both **1** and **2** that were recorded in the presence of copper(II) share some common features. For instance, their CD spectra are strongly pH dependent and no significant metal-ion binding is observed below pH 6. With the exception of the positive signal at about 230 nm observed in the copper(II)-**1** complex, the CD spectra of both peptide complexes, especially those at higher pH values, are characterised by negative ellipticity in the range of 215–220 nm and at 254 nm and positive ellipticity at 195–205 nm. In partic-

ular, the presence of the negative signal at 254 nm provides an indication of the similarity of the co-ordination environment that is experienced by the metal ion. The behaviour of this signal, which increases as the pH of the solution is raised, together with the sharp spectral changes that occur in the peptide region in the interval of pH 8–9, suggest that the complex species formed by both peptides at pH near neutrality are different from those present at basic pH values. Moreover, as $CuSO_4$ is titrated into a solution of peptide **1** at pH 7.3, the CD minimum at 216 nm reaches the maximum of intensity after the addition of one mole-equivalent of Cu^{II}. Further additions of Cu^{II} have no effect on the CD spectrum. A similar trend is observed for the CD minimum at 219 nm for the copper(II)-**2** complex at pH 9.2. These results indicate the presence of a single metal binding site in both peptides **1** and **2**.

The differences observed in the CD spectra collected between pH 7–8 for both peptide complexes may be attributed to the remaining part of the polipeptidic chain of **1** that, irrespective of the presence of Cu^{II}, contributes negative ellipticity in the range 195–200 nm due to its unordered conformation.

The CD data raise the possibility that both peptides can bind copper(II) in a similar fashion and allows the supposition that the negative band at 216 nm in the CD spectrum of the copper(II)-**1** complex is associated with the induction of a certain structure in the specific metal binding site which encompasses the HGGG sequence.

Electron spin resonance spectroscopy: The frozen ESR solution spectra of these complexes are characterised by the presence of signals from one complex species. Only when an excess of copper was used, a broad peak, due to the precipitation of copper hydroxide, was observed to slightly overlap the well-defined peaks associated with the complex species at pH 7. The magnetic parameters did not change on changing the metal–ligand (M/L) ratios, either with an excess of metal, up to an M/L = 5:1, or a two-fold excess of ligand. The parallel spin hamiltonian, the isotropic parameters and the optical visible data are reported in Table 1. These ESR spectra are characteristic of axial copper(II) complexes because $g_\parallel > g_\perp > 2.040$ (g factors); this suggests a $d_{x^2-y^2}$ or d_{xy} ground state in square planar, square pyramidal or tetragonal elongated octahedral stereochemistries.[22, 23] Raising the pH of the solution from neutral to basic reveals remarkable shifts in $g_\parallel$ and $A_\parallel$ (hyperfine coupling constant) as well as in the isotropic parameters. In particular, $g_\parallel$ decreases as $A_\parallel$ increases. An expansion of the coordination sphere of the copper complex or a replacement of oxygen donor atoms with nitrogen donor atoms could explain these shifts. This conclusion is further supported by the analysis of visible optical spectra, in which the usual blue shifts expected for successive deprotonation of peptidic nitrogen donor atoms of copper(II) complexes with di-, tri- or tetrapeptide ligands were observed with λ_{max} changing from 50 to 80 nm. Copper(II) complexes with different equatorial CuN_2O_2, CuN_3O or CuN_4 chromophores, in the case of amine, imidazole or only one amidic nitrogens,[1, 5, 24–26] show $g_\parallel$ ranging from 2.24 to 2.26 and $A_\parallel$ from 0.0180 to 0.0190 cm^{-1}. When considering CuN_4 chromophores in which two or more amide nitrogen donors are present,[6, 10d, 27] values of $g_\parallel = 2.17–2.19$ and $A_\parallel = 0.0190–0.0210$ cm^{-1} are easily obtained. In the case of these copper(II) complex species present in aqueous solution near neutrality, the relatively low hyperfine parallel constant leads us to consider a coordination polyhedron that experiences some kind of apical compression; this could be justified either by considering a square pyramidal geometry, an elongated octahedron with different donor atoms (oxygen or nitrogen donors) in the apical positions or a tetrahedral perturbation on a square based complex. In contrast, the lower $g_\parallel$ and the relative higher $A_\parallel$ values observed for the copper(II) complex species present at basic pH values could imply the in-plane coordination of four nitrogen donor atoms, either in a distorted square planar geometry or in an elongated octahedral stereochemistry with similar donor atoms (oxygen or nitrogen donors) in the apical sites. Unfortunately, low-temperature ESR spectra are lacking in superhyperfine (shf) structure; therefore, a complete attribution of the number of nitrogen donor atoms bound to equatorial copper plane is not possible. Very often, in these systems, the room-temperature ESR spectra show a strong dependence of the line-width on the nuclear quantum number of the transition, so that the high-field features generally present a clear superimposed shf pattern. As shown in Figure 5 and Figure 6, in the case of the neutral aqueous solutions of the copper(II) peptide complexes and in the case of the basic solutions of both complexes, well-defined 7-line or 9-line patterns were observed, respectively. Room-temperature ESR of the copper(II)-**1** system were noisier, because scans of these spectra were carried out at temperatures higher than 25 °C, but the 7-line and 9-line patterns are easily recognisable.

These spectroscopic data show that **1** and **2** have quite similar coordination sites, which are preserved with respect to other potential sites in the octapeptide chain. Different metal to ligand ratios, either an excess of ligand or an excess of metal, did not change the frozen solution ESR spectra of the complex species at neutral pH. These data can be easily rationalised by taking into account that, at neutral pH, three of the peptidic nitrogens of the polypeptide chain and the imidazole nitrogen atom could be involved in the coordination to

Table 1. Magnetic parameters obtained from ESR frozen solution spectra and room-temperature spectra for the copper(II) complexes with **1** and **2** on changing the pH of the aqueous solution.

pH	$g_\parallel$[a]	$A_\parallel$ [cm^{-1}][a]	λ_{max} [nm][a]	ε_{max} [$dm^3\ mol^{-1}\ cm^{-1}$][a]	g_{iso}[a]	A_{iso} [cm^{-1}][a]	A^N_{iso} [cm^{-1}][a]
Copper(II)-**1**							
6–8	2.235(4)	0.0160(2)	614(3)	105(5)	2.115(2)	0.0055(2)	0.0014(2)
10	2.193(2)	0.0190(2)	560(3)	96(5)	2.093(2)	0.0078(2)	0.0014(2)
Copper(II)-**2**							
7–8	2.231(4)	0.0153(2)	626(3)	71(5)	2.136(2)	0.0062(2)	0.0015(2)
10	2.178(2)	0.0194(2)	544(3)	86(5)	2.095(2)	0.0079(2)	0.0013(2)

[a] Presumed errors on the last digit are reported between parentheses.

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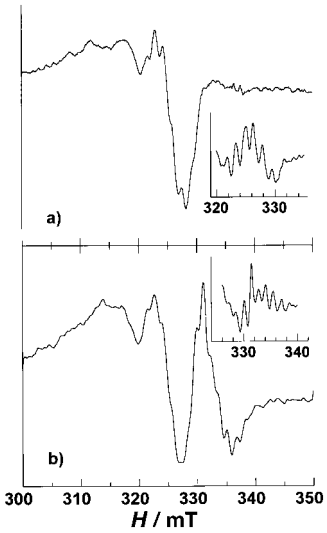

Figure 5. Room-temperature ESR spectra (320 K) of aqueous solutions of copper(II) complex species with octapeptide **1** at a) pH 7 and b) pH 10. Insets show the 2nd derivative mode of the highest field features. Instrumental settings: modulation frequency = 100 KHz; modulation amplitude = 3.3 – 4.2 G; time constant = 327 ms; sweep time = 2.8 min; receiver gain = $4-5\times10^5$; microwave power = 50 mW; microwave frequency = 9.4131 GHz.

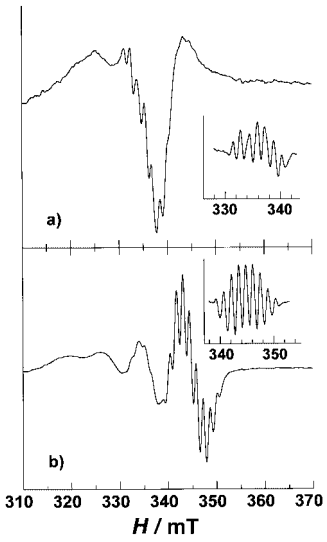

Figure 6. Room-temperature ESR spectra (293 K) of aqueous solutions of copper(II) complex species with tetrapeptide **2** at a) pH 7.3 and b) p 10. Insets show the 2nd derivative mode of the highest field features. Instrumental settings: modulation frequency = 100 KHz; modulation amplitude = 3.9 G; time constant = 327 ms; sweep time = 2.8 min; receiver gain = 4×10^4; microwave power = 50 mW; microwave frequency = 9.7668 GHz.

copper, giving rise to a square pyramidal geometry. The appearance of the well-resolved shf structure (7-line pattern) at room temperature may imply that the imidazole nitrogen atom is linked at one of the corners of the equatorial plane and that the in plane coordination is completed by an oxygen atom from a carbonyl group or a water molecule. In basic solution, deprotonation of other amide groups leads to the planar four coordination, which is demonstrated by the 9-line shf pattern.

The occurrence of tetrahedral perturbation cannot be ruled out on the basis of ESR spectral results alone, since similar values of high $g_{\|}$ and low $A_{\|}$ would be expected for tetrahedrally distorted geometries. Similarly, the visible optical spectra of copper are not very informative in this respect, as we have previously found similar λ_{max} and ε_{max} values for tetrahedrally perturbed copper systems.[5] Therefore, voltammetric measurements were carried out to test the physicochemical properties of these copper(II) complexes.

Cyclic voltammetry: These complexes undergo one-electron reduction processes which can be considered quasi-reversible (see Figure 7), because the peak-to-peak difference is larger

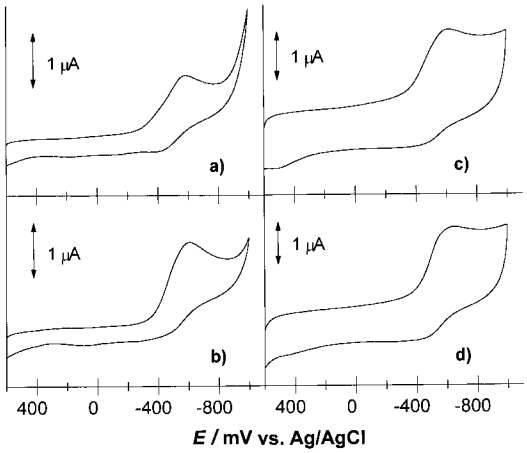

Figure 7. Cyclic voltammograms at 298 K of 0.5 mM copper(II)-**2** complex solutions at a) pH 7 and b) pH 10, and copper(II)-**1** at c) pH 7 and d) pH 10, recorded at a glassy carbon electrode with 100 mM KNO_3 ground electrolyte and a potential sweep rate of 200 mV s^{-1}.

than 60 mV,[28] but does not exceed 180 mV. Moreover, anodic peak to cathodic peak ratios were much lower than unity, which suggests that the reduction causes some kind of dissociation or stereochemical rearrangement as a consequence of the formation of copper(I) complex species. When working with an expanded range of potentials, anodic peaks at more positive potentials can be detected. As previously reported,[29] the presence of peptide nitrogen atoms in the coordination sphere of complexes strengthens the equatorial field, which stabilises the copper(II) complex and imposes a more rigid situation, one that generally does not meet the stereochemical requirements of copper(I) species. Table 2 reports the electrochemical data from the analysis of voltammograms of these complexes. The first point of comment is that negative redox potentials are found; these usually occur when typical tetragonally elongated octahedral, square planar or square-based pyramidal copper geometries are achieved. When strongly perturbed tetrahedral stereochemistries are found in copper chromophores that contain nitrogen and oxygen donor atoms, low negative or slightly positive redox potentials are obtained.[5, 6] Secondly, subtle differences are present in the redox potentials for the neutral and basic complex species in both systems. Generally, when copper(II) undergoes further complexation, peak potentials are shifted to the more negative region.[30] This slight difference probably

Table 2. E^o values versus NHE obtained by simulating the voltammetric curves, as explained in the Experimental Section, from cyclic voltammetric study of the copper(II) complexes with **1** and **2** on changing the pH of the aqueous solution together with values of the pertinent diffusion coefficients.

pH	ΔE_p[a] [mV]	i_a/i_c[a]	D[a] [$cm^2 s^{-1}$]	E^o_{exp}[a,b] [V vs. NHE]	E^o_{sim}[a,c] [V vs. NHE]
Copper(II)-**1**					
7	129(20)	0.17(3)	8.0×10^{-9}	−0.299(16)	−0.311(5)
10	175(8)	0.19(3)	8.0×10^{-9}	−0.308(23)	−0.340(10)
Copper(II)-**2**					
7	106(23)	0.26(6)	2.4×10^{-8}	−0.270(19)	−0.289(15)
10	108(31)	0.24(12)	2.4×10^{-8}	−0.299(6)	−0.311(14)

[a] Presumed errors on the last digit are reported between parentheses. [b] $E^o = \frac{1}{2}(E_{pc} + E_{pa})$. [c] Results obtained by the best fit procedure to the voltammetric curves using an EC mechanism and a k_s value of 8×10^{-3}.

comes from the substitution of water molecules or carbonyl oxygen atoms with peptide nitrogen donor atoms and the subsequent expansion of the coordination from five to six donor atoms. In other words, only little shifts of the peak potentials are detected because the metal is going from a quasi-fully coordinated to a fully coordinated situation.

Molecular geometry computations: Figure 8 shows some lowest energy stereochemistries selected among the various possibilities of linking copper to nitrogen donor atoms and

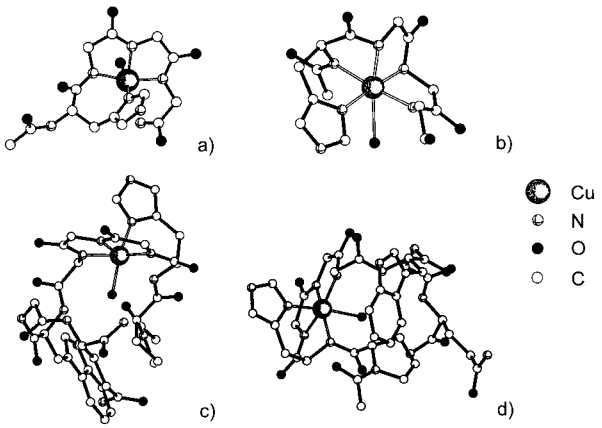

Figure 8. Optimised structural models of a) copper(II)-**2** complex species at neutral pH, b) copper(II)-**2** complex species at basic pH, c) copper(II)-**1** complex species at neutral pH, d) copper(II)-**1** complex species at basic pH.

oxygen donor atoms that come from carbonyl groups or water molecules. In copper(II)-**2** system, the geometry optimisation rapidly converges to a square-based pyramid with an equatorial plane formed by a water molecule, two peptidic nitrogen and one imidazole nitrogen donor atoms, with another peptidic nitrogen donor linked at the apical position as shown in Figure 8a. For the same copper(II) system, the complex species present at basic pH values presents an octahedral stereochemistry with an equatorial plane that has three peptidic nitrogen and one imidazole nitrogen donor atoms and two apical sites occupied by a peptidic nitrogen donor atom and a water molecule, as shown in Figure 8b. For the copper(II)-**1** system, the geometries of the complex species are analogous to those optimised for the copper(II)-**2** system, although the energies are slightly higher due to a bulkier ligand molecule (see Figure 8c and 8d). The involvement of carbonyl oxygen atoms in place of oxygen atoms from water molecules, or other peptidic nitrogen donor atoms cannot be ruled out, since these geometries only have a slightly higher energy.

Conclusion

The results of this study unequivocally demonstrate that octapeptide **1** has a unique site to which the copper(II) ion can coordinate. On the basis of CD and ESR results, the coordination of the copper(II) ion to both peptide **1** and **2** allows them to adopt a conformation in which structural identity somewhat similar to a β-turn is to be expected. In this site, the stereochemical arrangement of copper(II) can be described as a square-based pyramid with three nitrogen atoms and one oxygen atom at the edges of the equatorial plane and a further nitrogen atom apically linked. Moreover, the comparison with the results obtained from the copper(II) complex with tetrapeptide **2** excludes the possibility of eventual copper coordination to the pre-organised site formed by the sequence HGGG. The experiments which varied the ligand to metal ratios show the selectivity of this site. In fact, ligand excess does not modify the particular spectroscopic behaviour and an excess of copper was rejected as copper hydroxide. Therefore, at physiological pH, the participation of other donor atoms present in the octapeptide backbone or more than one ligand molecule can be excluded from the copper arrangement. Both spectroscopic and voltammetric data show that another peptidic nitrogen donor atom is involved only at higher pH, which may induce the expansion of the co-ordination sphere of the copper(II) complex from five to six donor atoms.

Finally, the redox potentials found for these copper(II) complexes are relatively negative. The redox process is quasi-reversible with a probable dissociation reaction as a consequence of the reduction of copper(II) complex, since the anodic peak intensity is much lower than that of the cathodic peak.

Experimental Section

Materials: All *N*-fluorenylmethoxycarbonyl (Fmoc)-protected aminoacids, NovaSyn-TGR resin, 2-(1-H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU), N-Hydroxybenzotriazole (HOBT) and N,N-dimethylformamide (DMF, peptide synthesis grade) were purchased from Novabiochem (Switzerland). *N,N*-diisopropylethyl amine (DIEA), triisopropylsilane (TIS), ethanditiole (EDT), thioanisole were purchased from Sigma/Aldrich and trifluoroacetic acid (TFA) from Carlo Erba. All other chemicals were of the highest available grade and were used without further purification.

Peptide synthesis and purification

Ac-PHGGGWGQ-NH_2 (1): The peptide was synthesised on a Milligen Model 9050 peptide synthesizer by using N^{α}-Fmoc (9-fluorenylmethoxycarbonyl) chemistry. The following amino acid derivatives were used: Fmoc-Gln(Trt)-OH, Fmoc-Gly-OPfp, Fmoc-His(Trt)-OH, Fmoc-Pro-OPfp, Fmoc-Trp-OH. The glutamine residue was linked to the NovaSyn-

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TGR resin by following the off-line procedure. The histidine and tryptophan residues were introduced according to the TBTU/HOBT/DIEA method. All other Fmoc-protected amino acids, were linked by the active ester method. After completion of the synthesis the N-terminal proline residue was acetylated with 0.3 M Ac_2O in DMF.

The peptide was cleaved from the resin by treatment with a mixture of TFA/phenol/H_2O/EDT/TIS/thioanisole (82.5:5.0:5.0:2.5:2.5:2.5 v/v) for 1.5 h. The solution containing the free peptide was filtered off from the resin, concentrated in vacuo at a temperature not exceeding 30 °C and then precipitated with cold freshly distilled diethyl ether. The precipitate was filtered, desiccated under vacuum, redissolved in 1% AcOH/H_2O solution and then lyophilised to yield 170 mg of crude peptide. Purification of the peptide was accomplished by semipreparative reversed-phase HPLC on a 250 × 10 mm Vydac C_{18} (5 μm particle size, 300 Å pores). The peptide was eluted isocratically with 12% acetonitrile/water/0.1% TFA at a flow rate of 3 mL min^{-1}. Elution profiles were monitored at 278 nm. The desired peptide fraction (10 min retention time) was collected, lyophilised and characterised by ^{1}H NMR spectroscopy and FAB-MS spectrometry [*m/z*: 836.4 $[M+H]^+$ calculated for $C_{37}H_{49}N_{13}O_{10}$ 835.37].

Ac-HGGG-NH$_2$ (2): This tetrapeptide was synthesised by condensation of the Ac-His-Gly-OH (100 mg; 0.39 mmol) fragment, previously obtained by the reaction of His-Gly with Ac_2O, with the Gly-Gly-NH$_2$ hydrochloride (63 mg, 0.37 mmol) dipeptide in DMF solution (5 mL) and in the presence of TBTU (125 mg, 0.39 mmol), HOBT (53 mg, 0.39 mmol) and DIEA (64 μL, 0.37 mmol). After completion of the synthesis the DMF was evaporated off, and the residue dissolved in water and loaded to a CM-Sephadex C-25 (NH_4^+ form) column (2 × 50 cm). The column was eluted initially with water (300 cm^3) and then with a linear gradient of aqueous NH_4HCO_3 (0–0.2 mol dm^{-3}; 1000 cm^3). Fractions were assayed by TLC and those containing the desired product (R_f = 0.1, eluent: BuOH/AcOH/H_2O 60:15:25 v/v; R_f = 0.3, eluent: PrOH/H_2O/EtOAc/NH_3 5:3:5:1 v/v) were combined, concentrated to dryness under vacuum at 40 °C, repeatedly dissolved in water and dried in order to let ammonium hydrogen carbonate decompose. The pure peptide (118 mg; 0.32 mmol; 87% yield) was then redissolved in water and lyophilised. The characterisation was achieved by means of ^{1}H NMR spectroscopy and FAB-MS spectrometry [*m/z*: 368.2 $[M+H]^+$ calculated for $C_{14}H_{21}N_7O_5$ 367.16].

Spectroscopic measurements

ESR spectroscopy: Frozen solution ESR spectra were recorded on a conventional Bruker ER 200 D spectrometer driven by the ESP 3220 data system and equipped with a standard low temperature unit, ER 4111 VT. Copper(II)-complex solutions (1 mM) were prepared in situ by mixing a standard solution of $^{63}Cu(NO_3)_2$ with solutions of the tetrapeptide **2** or the octapeptide **1**, in various metal to ligand ratios, ranging from M/L = 1:2 to M/L = 5:1, and adjusting the pH of the resulting solution to the values of 7–8 or 9–10 by adding KOH 10 mM. Methanol (less than 10% of the total volume) was added to the aqueous copper(II) complex solutions to increase resolution, which is poor in aqueous solutions. Room-temperature ESR spectra were obtained by using a Bruker quartz aqueous solution flat cell or glass capillaries, which were inserted in quartz tubes, to run measurements in the 293–323 K temperature range. Any spectral features from the glass capillaries were first recorded and then subtracted from the pertinent copper complex spectrum. High molecular mass species have low tumbling rates so superhyperfine structure can be difficult to observe. Since the line-width parameters are function of g and A anisotropy as well as of the correlation time, which depends on the viscosity of the solution, increasing the temperature decreases the viscosity of aqueous solution and increases the tumbling rates.[31] Therefore, superhyperfine structure attributable to three or four nitrogen donor atoms could be resolved in the case of the copper(II)-**1** system. Frozen solution spectra revealed signals coming from two different species present in this system, the first at pH near neutrality and the second at basic pH values. Parallel spin hamiltonian parameters from frozen solution spectra were obtained directly from the experimental spectra and were calculated from the 2nd and 3rd lines in order to remove second-order effects.[32] Room-temperature spectra were analysed by generating computed spectra employing a computer program, substantially devised by J. R. Pilbrow.[33] It was necessary to use line-width parameters that depended on the nuclear quantum number of the transition. Geometry optimisation was carried out by making use of MOMEC[34, 35] with an adapted force field in conjunction with HYPERCHEM[36] in order to gain an idea of the possible geometries of these complexes. The results were compared with the information obtained by the interpretation of the ESR spectra of each copper(II) complex species, in order to see if these were consistent.

Visible optical spectroscopy: Visible optical spectra were performed with a diode-array Hewlett-Packard 8452A spectrophotometer on copper(II) aqueous solutions (1 mM) contained in 1 cm quartz cells in the same pH range.

Circular dichrosim: The CD spectra were obtained at 25 °C under a constant flow of nitrogen on a Jasco J-600 spectropolarimeter, which was calibrated with an aqueous solution of ammonium D-camphorsulphate.[37] Experimental measurements were carried out in water and at different pH values by using a 1 mm pathlength cuvette. The CD spectra pertinent to the free peptide ligands were recorded in the UV region (190–260 nm), whereas those in the presence of Cu^{II} were examined in the wavelength range of 190–380 nm. The spectra represent the average of 8–20 scans. CD intensities are expressed as mean residue ellipticity (deg cm^2 dmol^{-1}) calculated by $[\theta] = \theta/lcn$, where θ is the ellipticity observed (mdeg), l is the pathlength of the cell (cm), c is the peptide concentration (M) and n is the number of peptide bonds in the sequence.

Cyclic voltammetry: Cyclic voltammograms of the copper(II) complexes in aqueous solution (100 mM KNO_3 as ground electrolyte) were obtained by means of a BAS CV-50W voltammetric analyser equipped with a C3 cell stand driven by a standard PC. Copper(II) complexes (0.5 mM) at neutral and basic pH values were analysed at 25 ± 0.1 °C in a 5 mL BAS glass cell with a three-electrode assembly. A glassy carbon electrode was used as the working electrode (1 mm diameter), a platinum electrode as counter and a standard Ag/AgCl electrode as reference. Aqueous complex solutions were degassed by using ultra-pure nitrogen, which had been passed over copper wires heated at 400 °C. To analyse these voltammograms, an ESP (electrochemical simulation package, version 2.4) program was used,[38] which assumes that the one-electron redox behaviour can be accounted for an EC mechanism (k_s = 0.008 cm s^{-1}).[39] E^o values calculated this way were referred to NHE (normal hydrogen electrode), and compared with the experimental values obtained by $(E_{pc} + E_{pa})/2$, which takes account that the $E^o_{Ag/AgCl}$ versus $E^o_{NHE\ (water)}$ is +0.223 V at 25 °C.[40] This latter value was also checked using the methylviologen couple (MeV^{2+}/MeV^+, $E^o = -0.446$ V versus NHE).[41]

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[1] G. Arena, R. P. Bonomo, G. Impellizzeri, R. M. Izatt, J. D. Lamb, E. Rizzarelli, *Inorg. Chem.* **1987**, *26*, 795.
[2] G. Arena, R. P Bonomo, L. Casella, M. Gullotti, G. Impellizzeri, G. Maccarrone, E. Rizzarelli, *J. Chem. Soc. Dalton Trans.* **1991**, 3203.
[3] R. P. Bonomo, F. Bonsignore, E. Conte, G. Impellizzeri, G. Pappalardo, R. Purrello, E. Rizzarelli, *J. Chem. Soc. Dalton Trans.* **1993**, 1295.
[4] G. Arena, G. Impellizzeri, G. Maccarrone, G. Pappalardo, E. Rizzarelli, *J. Chem. Soc. Dalton Trans.* **1994**, 1227.
[5] R. P. Bonomo, E. Conte, G. Impellizzeri, G. Pappalardo, R. Purrello, E. Rizzarelli, *J. Chem. Soc. Dalton Trans.* **1996**, 3093.
[6] R. P. Bonomo, G. Impellizzeri, G. Pappalardo, R. Purrello, E. Rizzarelli, G. Tabbì, *J. Chem. Soc. Dalton Trans.* **1998**, 3851.
[7] G. Impellizzeri, G. Pappalardo, R. Purrello, E. Rizzarelli, A. M. Santoro, *Chem. Eur. J.* **1998**, *4*, 1791.
[8] a) J. P. Schneider, J. W. Kelly, *Chem. Rev.* **1995**, *95*, 2169–2187; b) T. Sasaki, M. Lieberman in *Comprehensive Supramolecular Chemistry*, *Vol. 4* (Ed.: Y. Murakami), Elsevier Science, **1996**, pp. 193–242.
[9] See for example: a) T. Handel, W. F. Degrado, *J. Am. Chem. Soc.* **1990**, *112*, 6710; b) J. T. Jr. Kellis, R. J. Todd, F. H. Arnold, *Bio/Technol.* **1991**, *9*, 994; c) R. J. Todd, M. E. Van Dam, D. Casimiro, B. L. Haymore, F. H. Arnold, *Proteins: Struct. Funct. Genet.* **1991**, *10*, 156; d) S. Suh, B. L. Haymore, F. H. Arnold, *Protein Eng.* **1991**, *4*, 301; e) A. Pessi, E. Bianchi, A. Crameri, S. Venturini, A. Tramontano, M.

Sollazzo, *Nature* **1993**, *362*, 367; f) D. J. Matthews, J. A. Wells, *Chem. Biol.* **1994**, *1*, 25; g) B. Pierret, H. Virelizier, C. Vita, *Int. J. Pept. Protein Res.* **1995**, *46*, 471; h) C. Vita, C. Roumestand, F. Toma, A. Menez, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 6404; i) S. Yilmaz, M. Widersten, T. Emahazion, B. Mannervik, *Protein Eng.* **1995**, *8*, 1163; l) L. Regan, *Trends Biochem. Sci.* **1995**, *20*, 280; m) J. Voss, W. L. Hubbel, H. R. Kaback, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 12300; n) E. Farinas, L. Regan, *Protein Sci.* **1998**, *7*, 1939; o) S. K. Wilcox, C. D. Putnam, M. Sastry, J. Blankenskip, W. J. Chazin, D. E. McRee, D. B. Goodin, *Biochemistry* **1998**, *37*, 16853; p) S. F. Marino, L. Regan, *Chem. Biol*. **1999**, *6*, 649.

[10] See for examples:a) B. Imperiali, T. M. Kapoor, *Tetrahedron* **1993**, *49*, 3501; b) J. P. Schneider, J. W. Kelly, *J. Am. Chem. Soc*. **1995**, *117*, 2533; c) R. P. Cheng, S. L. Fisher, B. Imperiali, *J. Am. Chem. Soc.* **1996**, *118*, 11349; d) R. P. Bonomo, L. Casella, L. De Gioia, H. Molinari, G. Impellizzeri, T. Jordan, G. Pappalardo, R. Purrello, E. Rizzarelli, *J. Chem. Soc. Dalton Trans.* **1997**, 2387; e) W. D. Kohn, C. M. Kay, B. D. Sykes, R. S. Hodges, *J. Am. Chem. Soc*. **1998**, *120*, 1124; f) O. Hènin, B. Barbier, F. Boillot, A. Brack, *Chem. Eur. J.* **1999**, *5*, 218.

[11] a) L. M. Sayre, G. Perry, M. A. Smith, *Curr. Opin. Chem. Biol*. **1999**, *3*, 220; b) D. J. Waggoner, T. B. Bartnikas, J. D. Gitlin, *Neurobiol. Dis.* **1999**, *6*, 221.

[12] a) F. Edenhofer, S. Weiss, E.-L. Winnacker, M. Famulok, *Angew. Chem*. **1997**, *109*, 1748; *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 1674, and references cited therein; b) S. B. L. Ng, A. J. Doig, *Chem. Soc. Rev.* **1997**, *26*, 425.

[13] a) D. R. Brown, K. Qin, J. W. Herms, A. Madlung, J. Manson, R. Strome, P. E. Fraser, T. Kruck, A. von Bohlen, W. Schulz-Schaeffer, A. Giese, D. Westaway, H. Kretzschmar, *Nature* **1997**, *390*, 684; b) J. Stöckel, J. Safar, A. C. Wallace, F. E. Cohen, S. B. Prusiner, *Biochemistry* **1998**, *37*, 7185.

[14] a) D. R. Brown, W. J. Schulz-Schaeffer, B. Schmidt, H. A. Kretzschmar, *Exp. Neurol.* **1997**, *146*, 104; b) D. R. Brown, B. Schmidt, H. A. Kretzschmar, *J. Neurochem.* **1998**, *70*, 1686; c) D. R. Brown, A. Besinger, *Biochem. J.* **1998**, *334*, 423; d) D. R. Brown, B.-S. Wong, F. Hafiz, C. Clive, S. J. Haswell, I. M. Jones, *Biochem. J.* **1999**, *344*, 1; e) J. Herms, T. Tings, S. Gall, A. Madlung, A. Giese, H. Siebert, P. Schürmann, O. Windl, N. Brose, H. A. Kretzschmar, *J. Neurosci.* **1999**, *19*, 8866.

[15] a) R. H. Kimberlin, G. C. Millson, L. Bountiff, S. C. Collis, *J. Comput. Pathol.* **1974**, *84*, 263; b) I. H. Pattison, J. N. Jebbett, *Res. Vet. Sci.* **1971**, *12*, 378.

[16] a) D. McKenzie, J. Bartz, J. Mirwald, D. Olander, R. Marsh, J. Aiken, *J. Biol. Chem*. **1998**, *273*, 25545; b) J. D. F. Wadsworth, A. F. Hill, S. Joiner, G. S. Jackson, A. R. Clarke, J. Collinge, *Nat. Cell. Biol.* **1999**, *1*, 55.

[17] a) M. P. Hornshaw, J. R. McDermott, J. M. Candy, J. H. Lakey, *Biochem. Biophys. Res. Commun*. **1995**, *214*, 993; b) T. Miura, A. Hori-i, H. Takeuchi, *FEBS Lett.* **1996**, *396*, 248.

[18] a) J. H. Viles, F. E. Cohen, S. B. Prusiner, D. B. Goodin, P. E. Wright, H. J. Dyson, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 2042; b) T. Miura, A. Hori-i, H. Mototani, H. Takeuchi, *Biochemistry* **1999**, *38*, 11560.

[19] A. Perczel, M. Hollósi, in *Circular Dichroism and the Conformational Analysis of Biomolecules* (Ed.: G. D. Fasman), Plenum, New York, **1996**, pp. 295 – 380

[20] R. W. Woody, *Biopolymers* **1978**, *17*, 1451.

[21] J. M. Tsangaris, J. Wen Chang, R. B. Martin, *J. Am. Chem. Soc*. **1969**, *91*, 726.

[22] B. J. Hathaway, D. E. Billing, *Coord. Chem. Rev.* **1970**, *5*, 143.

[23] B. J. Hathaway, A. A. G. Tomlinson, *Coord. Chem. Rev.* **1970**, *5*, 1.

[24] R. P. Bonomo, R. Calì, V. Cucinotta, G. Impellizzeri, E. Rizzarelli, *Inorg. Chem.* **1986**, *25*, 1641.

[25] R. P. Bonomo, F. Riggi, A. J. Di Bilio, *Inorg. Chem.* **1988**, *27*, 2510.

[26] R. P. Bonomo, S. Pedotti, G. Vecchio, E. Rizzarelli, *Inorg. Chem.* **1996**, *35*, 6873.

[27] R. P. Bonomo, E. Conte, R. Marchelli, A. M. Santoro, G. Tabbì, *J. Inorg. Biochem.* **1994**, *53*, 127.

[28] A. M. Bond in *Modern Polarographic Methods in Analytical Chemistry,* Marcel Dekker, New York and Basel, **1980**, p. 182 – 190.

[29] R. P. Bonomo, R. Marchelli, G. Tabbì, *J. Inorg. Biochem.* **1995**, *60*, 205.

[30] D. R. Crow, *Polarography of Metal Complexes*, Academic Press, London and New York, **1969**, Chapter 4.

[31] B. A. Goodman, J. B. Raynor in *Advances in Inorganic Chemistry and Radiochemistry*, *Vol. 13* (Eds.: H. J. Emeleus, A. G. Sharpe), Academic Press, New York, **1970**, pp.135 – 362.

[32] A. Lund, T. Vanngard, *J. Chem. Phys.* **1969**, *50*, 2979.

[33] J. R. Pilbrow, M. E. Winfield, *Mol. Phys.* **1973**, *26*, 1073.

[34] P. Comba, T. V. Hambley, N. Okon, *MOMEC*, *A Molecular Modeling Package for Inorganic Compounds*, LawerOkon, Heidelberg, Germany, **1995**, serial no. 5761-33456-7657.

[35] P. Comba, P. V. Bernhardt, *Inorg. Chem.* **1992**, *31*, 2638.

[36] *Hyperchem release 5.1*, Hypercube , license no. 500-10002406.

[37] Chen, G. C., Yang, J. T., *Anal. Lett*. **1977**, *10*, 1195.

[38] Copyright by Professor Carlo Nervi, this package can be downloaded at the internet address http://www.lem.ch.unito.it/chemistry/electrochemistry.html.

[39] A. J. Bard in *Electrochemical Methods: Fundamentals and Applications*, Wiley, **1980**, p. 430.

[40] D. T. Sawyer, J. L. Roberts in *Experimental Electrochemistry for Chemists,* Wiley, **1974**, p. 42.

[41] T. Kuwana, *ACS Symp. Ser.* **1977**, *38*, 943.

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  0947-6539/00/0622-4202 $ 17.50+.50/0