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New Copper(II)/Cyclic Tetrapeptide System That Easily Oxidizes to Copper(III) under Atmospheric Oxygen

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A 13-membered ring cyclic tetrapeptide was synthesized by the solid-phase peptide synthesis method, and its copper(II) coordination properties were analyzed by optical spectroscopy, mass spectrometry, and electrochemistry. All collected data strongly support the presence, at alkaline pH, of a stable peptide/copper(III) complex that is formed in solution by atmospheric dioxygen oxidation. On the basis of previous studies on cyclic peptide/copper systems, we suggest that the copper(III) ion is at the center of the ligand's cavity being coordinated to four deprotonated amide nitrogen atoms. This donor set would greatly lower the redox potential for the Cu^{III}/Cu^{II} couple, thus allowing easy oxidation of the coordinated copper(II) by atmospheric oxygen.

Figure 1. *c*(Lys-D-His-*β*-Ala-His).

In the frame of a larger research project aimed at preparing and characterizing linear and cyclic oligopeptides as selective copper(II) ligands, we recently described the synthesis of a few cyclic tetrapeptides bearing imidazole side chains and analyzed, in detail, their copper(II) binding properties in aqueous solution.[1] The copper(II) species obtained are of interest in relation to copper-protein active-site biomimetics. The 12-membered cyclic peptides investigated so far in our laboratory turned out to behave as relatively strong copper(II) ligands, giving rise to various types of copper(II) donor sets (from NO_3 to N_4) depending upon the pH. As the pH is raised, an increasing number of backbone nitrogen atoms are progressively engaged in copper(II) coordination. In no case, even under fully aerobic conditions, was spontaneous oxidation of copper(II) to copper(III) ever observed. Subsequently, our studies have been directed toward the synthesis of homologous peptide lig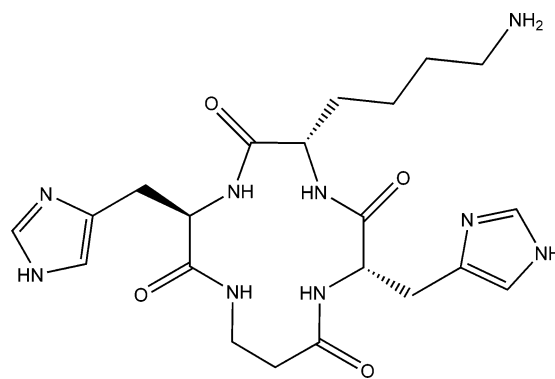ands, in particular cycles of greater size (for instance, 13- and 14-membered cycles), in order to specifically assess the effects of an enlarged metal binding cavity on the copper binding properties. The syntheses were performed by solid-phase anchoring of the His side chain to a trityl-type resin and use of Fmoc/ʹBu/allyl orthogonal protection.[2] The amino acid residues were introduced according to the HOBt/TBTU/NMM procedure. Taking advantage of the pseudodilution phenomenon, on-resin cyclization was performed with TBTU/DIPEA at room temperature, with no evidence of byproducts resulting from dimerization and/or oligomerization.[2] The *c*(Lys-D-His-*β*-Ala-His) ligand (DK13 hereafter; Figure 1) manifested a very peculiar and unexpected reactivity toward copper(II) ions.

Indeed, the addition of a stoichiometric amount of copper(II) sulfate to a DK13 solution, adjusted to pH 8 by NaOH, resulted in the progressive development of a very intense and unexpected orange color. The color intensity was even higher at pH 12. This process was investigated by UV−vis spectroscopy. Typical spectrophotometric profiles for DK13/copper(II) samples, taken at various pH values, are shown in Figure 2. At pH 12.2, two main bands are detected at about 250 and 330 nm ($\epsilon \cong 9840$ and 5580 cm^{-1} M^{-1}, respectively), while a very weak absorption at around 500

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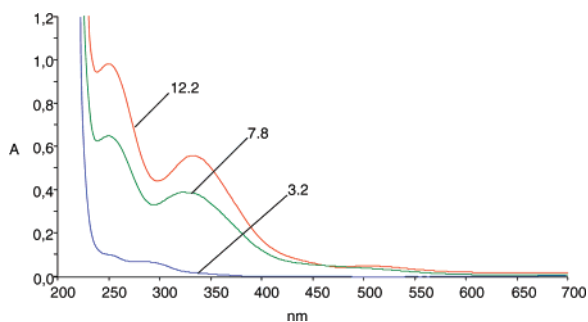
Figure 2. UV−vis spectrum of the system DK13/copper(II) at increasing pH values. The UV maxima corresponding to the copper(III) formation are shown (DK13, 0.15×10^{-3} M; CuSO$_4$, 0.1×10^{-3} M). pH values are specified on the profiles.
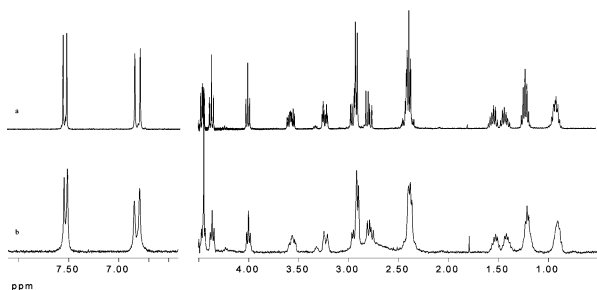

Figure 3. ^{1}H NMR spectrum (D$_2$O, pH 12.5): (a) DK13 ligand; (b) DK13/CuSO$_4$ = 3/1 (DK13, 6.0×10^{-3} M). Spectra were recorded on a Varian 400 MHz Mercury Plus at room temperature.

nm indicates the persistence of a residual amount of a copper-(II) species. We quickly realized that the development of the above characteristic spectral pattern was critically dependent on the presence of atmospheric oxygen: as a matter of fact, the bands at 250 and 330 nm did not appear when working under strict anaerobic conditions. We also observed that the process is pH-dependent, being stressed by the highest pH values (Figure 2). When the spectrum of the same sample was recorded after 24 h (room temperature; pH = 12.2), the two main absorption maxima looked substantially unmodified, with just a small decrease in the intensity, implying a substantial stability for the obtained species.

On the basis of previous literature, we hypothesized that the above spectrophotometric behavior might be related to progressive oxidation of copper(II) to copper(III), induced by atmospheric oxygen and favored by copper coordination to four deprotonated amide nitrogen donors, within a roughly square-planar chromophore.[3] Indeed, square-planar copper-(III) d^8 centers are known to exhibit intense ligand-to-metal charge-transfer bands in the UV region,[4] as is the case here. Notably, ^{1}H NMR spectra, recorded on the orange solution, showed four narrow signals of the imidazole protons. The entire proton pattern, collected at low ligand-to-copper(II) ratio, did not suffer from significant line broadening at variance with previously reported cases (Figure 3). This behavior is suggestive of nearly quantitative oxidation of paramagnetic copper(II) to diamagnetic copper(III).

The ^{1}H NMR spectrum of the same sample taken after 1 h did not reveal any significant change, implying a substantial stability of the copper(III) species. Accordingly, the X-band electron paramagnetic resonance (EPR) spectrum (290 K) recorded on a freshly prepared DK13/copper(II) sample,
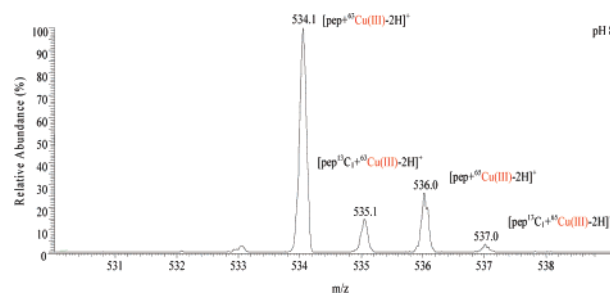

Figure 4. ESI(+)-MS spectra of a 1:1 DK13/copper(II) solution at pH 8.

brought at pH 12 (1.2:1 molar ratio), did not show any residual copper(II) signal witnessing a rapid and nearly complete oxidation to the corresponding EPR-silent copper(III) species. Positive-mode electrospray ionization mass spectrometry ESI(+)-MS measurements were carried out on methanolic equimolar (5×10^{-4} M) DK13/copper solutions at different pH values (Figure 4).[5] It is noteworthy that, independently of the pH of the solution, only monoprotonated species could be detected, while highly charged ions were not formed.

Under basic conditions, the most abundant ions in the m/z 530−540 region are seen at m/z 534 (Figure 4). This species differs by 1 mass unit from the expected species at m/z 535 assigned to [L-Cu^{II}-H]$^+$. This shift of 1 mass unit suggests that the measured positive species has a proton that is less than expected, thus indicating an almost quantitative oxidation of copper(II) to copper(III). Tandem mass spectrometry has been used to obtain information about copper coordination in the complexes. The most evident decomposition shown by the [L-Cu^{III}-2H]$^+$ species is assigned to the elimination of an ammonia molecule (see Figure 3S in the Supporting Information). Abundant ions attributable to the elimination of one or more aminoacidic residues were not detected, favoring the presence of strong interactions between the metal center and the DK13 ligand.

The physicochemical results reported so far point out quite unambiguously that the formed orange product is truly a copper(III) species resulting from progressive and rapid aerobic oxidation of copper(II) to copper(III). The process is spontaneous under normal aerobic conditions, suggesting that the DK13 ligand strongly stabilizes the oxidation state 3+ of the bound copper. To further define such a stabilization, electrochemical and spectroelectrochemical measurements were carried out. A freshly prepared mixture of DK13 and CuSO$_4$ in a dilute NaOH solution (pH 12) under a nitrogen atmosphere affords a substantially colorless solution that does not exhibit any response in cyclic voltammetry. This means that the strong coordination of the copper(II) ion to the DK13 ligand shifts the copper(II) reduction beyond the cathodic window of the alkaline solution. In fact, a CuSO$_4$ solution at pH 6.5 undergoes the Cu^{II}/Cu^I/Cu^0 sequence at +0.15 and +0.01 V, respectively, and exhibits, in the reverse

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(5) Preliminary results presented by: Giorgi, G.; Ginanneschi, M.; Messori, L. 55th ASMS Conference, June 3−7, 2007, Indianapolis, IN; Poster ThP 082.
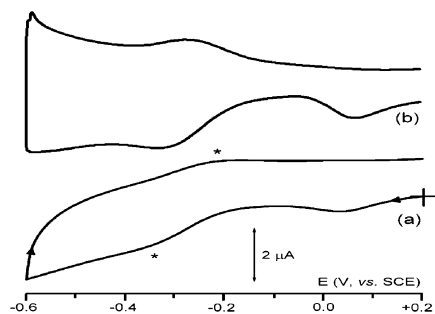
Figure 5. Cyclic (a) and first derivative deconvoluted (b) voltammograms recorded at a glassy carbon electrode in a NaOH solution (pH 12) containing CuSO$_4$ (0.6×10^{-3} M) and DK13 (0.7×10^{-3} M). Scan rate: 0.05 V s^{-1}.

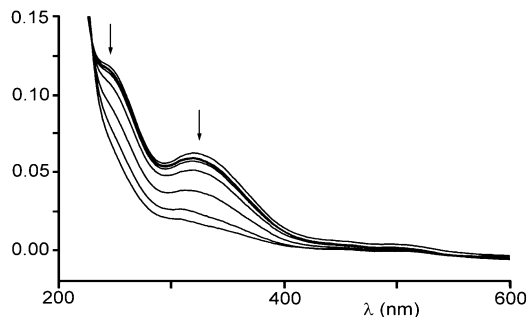**Figure 6.** UV−vis spectral trend recorded in an optically transparent thin-layer electrode cell upon progressive reduction ($E_w = -0.4$ V vs Ag wire pseudoreference electrode) of an aqueous solution (pH 12) containing CuSO$_4$ (0.6×10^{-3} M) and DK13 (0.7×10^{-3} M) after its exposition at air for 24 h.

scan, the anodic stripping of electrodeposited copper metal at +0.24 V (vs SCE). Upon standing in air, the DK13/CuSO$_4$ solution progressively turns orange, affording, after 24 h, the voltammetric profiles shown in Figure 5. A poorly resolved reduction process in cyclic voltammetry (asterisked peak system) but sufficiently well defined in deconvoluted voltammetry appears ($E^{\circ\prime} = -0.04$ V vs NHE), which features partial chemical reversibility. Because no anodic stripping is present in the reverse scan, it seems reasonable to attribute the actual cathodic process to the Cu^{III}/Cu^{II} couple.

A similar, poorly resolved, cyclic voltammetric pattern was earlier found for the $c(\beta$-Ala-Gly-β-Ala-Gly)/Cu^{III} complex, which was prepared by anodic oxidation of the related copper(II) complex.[6] For a better definition of the extent of chemical reversibility of the Cu^{III}/Cu^{II} reduction in our system, we recorded the spectroelectrochemical trend of DK13/copper(III) at pH 12 upon stepwise reduction (Figure 6), followed by stepwise reoxidation. Notably, the two main bands ($\lambda_{max} = 245$ and 324 nm, respectively) progressively decrease, but upon step-by-step reoxidation ($E_w = +0.2$ V), the original spectrum is restored. This characteristic behavior as well as the presence of a well-defined isosbestic point at 220 nm testifies to the chemical reversibility of the Cu^{III}/Cu^{II} interconversion.

This means that the apparent partial chemical reversibility detected in cyclic voltammetry most likely arises from electrode surface phenomena. Previous studies reported that the Cu^{II}/Gly$_4$ solutions are autoxidized by dioxygen uptake at pH 7−10 through the formation of an autocatalytic Cu^{III}(H$_{-3}$Gly$_4$)$^-$ complex.[7] In any case, a copper(III)-coordinated species could only be obtained by chemical or electrochemical oxidation.[8] At variance, the present DK13/copper(II) system is nearly quantitatively oxidized to copper-(III) by atmospheric oxygen at room temperature. Because this oxidation process is highly favored by strong alkaline conditions, it is well conceivable that the stabilization observed arises from copper coordination to four deprotonated amide nitrogen atoms, thus generating a [Cu^{III}(H$_{-4}$L)]$^-$ species. Margerum et al. stated for their peptide ligands[9] that N$^-$ ions from the deprotonated peptide backbone are the strongest σ donors in copper/peptide complexes and can stabilize a copper(III) species. We can assume that copper(III) ions may accommodate the ligand's cavity, thus affording a square-planar complex. Notably, the stabilization effects brought about by our ligand are far higher than those reported by Margerum et al. for the related 14-membered peptide $c(\beta$-Ala-Gly-β-Ala-Gly). As a matter of fact, the formal electrode potential of the DK13/copper(III,II) system is lower (by about 0.4 V) than that reported for $c(\beta$-Ala-Gly-β-Ala-Gly) at alkaline pH, probably reflecting the thermodynamic stabilization triggered by the higher planarity imposed by the present 13-membered macrocycle. To better understand the role of the His side chains in the overall reaction, we prepared the new ligand c(Lys-Gly-β-Ala-Gly) (GK13), where the two histidines are replaced by two glycines. Upon the addition of an equimolar amount of CuSO$_4$ at pH 12, a light-blue precipitate soon formed. Ultraperformance liquid chromatography MS (UPLC-MS) inspection of the remaining aqueous solution did not reveal the presence of the GK13 ligand, while UV−vis spectrum showed some very weak absorptions at about 420 and 340 nm, again suggesting the presence of a diluted copper(III) complex. Thus, in view of the highly different behavior shown by GK13 in comparison to DK13, we can say that the presence of the histidine ligands is crucial to ensure complete occurrence of the copper(II) to copper-(III) oxidation reaction and solubilization of the resulting copper(III) species. Work is in progress to determine the three-dimensional structure of the DK13/copper(III) orange complex and to further evaluate the behavior of GK13.

Supporting Information Available: Synthesis, purification, and analytical data for DK13 and GK13, MS/MS analysis under basic conditions, and electrochemical, spectroelectrochemical, EPR, and UV−vis spectroscopy operative conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

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