J|A|C|S COMMUNICATIONS

Published on Web 05/04/2009

# A Cysteine-Based Tripodal Chelator with a High Affinity and Selectivity for Copper(I)

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Metal overload plays an important role in several diseases and intoxications.[1] Among these metals, copper (Cu) is an essential element which is used as a cofactor in many redox proteins, which are involved in several vital processes. Free Cu can also promote Fenton-like reactions and thus is very toxic even at low concentrations. Therefore, the intracellular Cu concentration needs to be rigorously controlled to ensure that it is provided only to the essential enzymes and does not accumulate to toxic levels.[2] For instance, Wilson's disease is one of the major genetic disorders of Cu metabolism in humans. Failure of the copper transport in hepatocytes results in cytosolic Cu accumulation with associated cellular injury.[3,4] Cu is also involved in neurodegenerative diseases such as Alzheimer's disease and is suspected to cause amyloid $\beta$ precipitation and toxicity.[5,6] Therefore, as chelation therapy[1,4,5] is currently used or proposed for treating these disorders, it is of major interest to develop molecules able to efficiently and selectively bind Cu. Biological molecules that traffic or sequester Cu in cells are good models for designing efficient Cu chelators. As the cytoplasm of most eukaryotic cells is a reducing environment, the predominant oxidation state of Cu in cells is Cu(I). Many intracellular Cu transporters contain a conserved N-terminal MxCxxC sequence that binds metal ions with two cysteines.[7] In a previous report, we demonstrated that this sequence is highly selective for Cu(I) over the essential ion Zn(II) that could compete in cells.[8,9] Metallothioneins (MTs) are other proteins able to complex Cu. Indeed, when Cu is in excess of physiological requirements, cells induce the biosynthesis of these small cysteine-rich proteins, which form Cu(I) clusters.[10]

We decided to take advantage of the high affinity of cysteine sulfur donors for Cu(I) evidenced in living organisms to design efficient and selective copper chelators. For that purpose, several cysteines were attached with peptide bonds to nonbiological scaffolds to obtain pseudopeptide ligands.[11] Polyaminocarboxylate spacers are attractive because they provide a series of chemical scaffolds with a range of carboxylic acid numbers that can be easily functionalized with cysteines. In this communication, we present a $C_3$-symmetric ligand anchored on a nitrilotriacetic acid (NTA) moiety extended by three converging metal-binding cysteine chains. We demonstrate that this tripodal ligand has a high affinity and selectivity for Cu(I).

The tris(cysteine) pseudopeptide ligand $LH_3$ was synthesized by coupling the triacid NTA with the free amine function of an O,S-protected derivative of cysteine, HCys(Trt)OEt, in presence of classical peptide-synthesis coupling reagents, followed by deprotection of the thiol groups under acidic conditions [see the Supporting Information (SI) for detailed experimental procedures]. Binding of Cu(I) was investigated by UV spectroscopy at pH 7.4. Addition of aliquots of Cu(I) to the ligand solution resulted in the appearance of a band centered at 262 nm that linearly increased with increasing Cu(I) concentration up to 2 equiv (Figure 1). This band is characteristic of ligand-to-metal charge-transfer (LMCT)

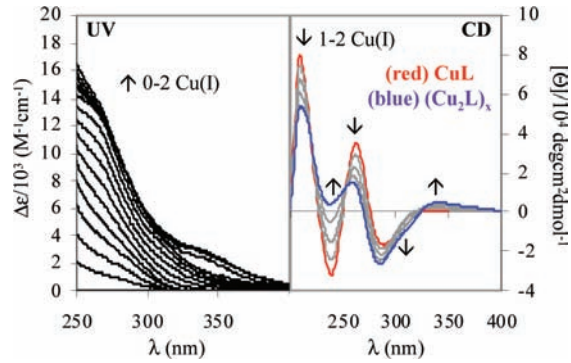
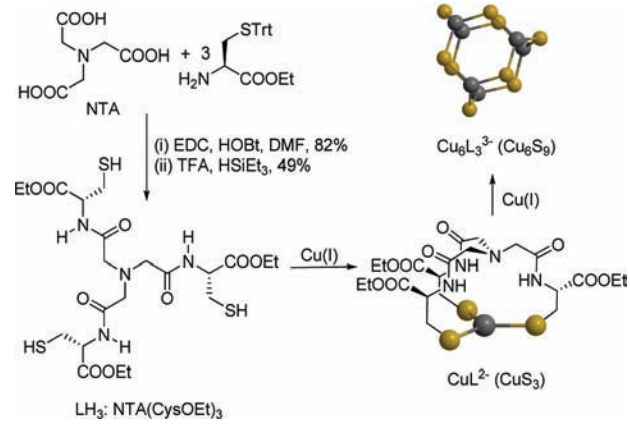



***Figure 1.*** UV and CD titrations of L (~50 $\mu$M) with Cu(I) at pH 7.4 [9:1 (v/v) 20 mM phosphate buffer/$CH_3CN$].

transitions of the thiolate−Cu(I) bonds in a $(Cu_2L)_x$ complex. The extinction coefficient of this LMCT band is 13 000 $M^{-1}$ $cm^{-1}$, which is compatible with the values of ~7000 $M^{-1}$ $cm^{-1}$ per bound Cu found in MTs.[12] The examination of the shoulder at 340 nm indicated a more complicated behavior, namely, the formation of the two complexes represented in Scheme 1, which were investigated with complementary experimental methods.

***Scheme 1***




The $C_3$-symmetric mononuclear complex CuL is evidenced in the $^1$H NMR spectra of L with less than 1 equiv of Cu(I) (Figure S2 in the SI). In this complex, the $\beta$-protons of the cysteines and the apical $NCH_2$ protons give well-resolved AB doublets having large chemical shift differences with respect to the free ligand ($\Delta\delta$ = 0.05 and 0.04 ppm for L, 0.47 and 0.91 ppm for CuL), suggesting that the ligand adopts a rigid conformation with the three thiolate arms wrapped around the metal ion. The exchange between the free ligand and this complex is very slow, as evidenced by the absence of exchange correlation in the 2D exchange spectroscopy (EXSY) spectrum. The diffusion coefficients were measured by



10.1021/ja901700a CCC: $40.75 

pulsed-gradient spin-echo (PGSE) NMR spectroscopy to infer the species molecularity in solution.[13] In 9:1 (v/v) water (pD 7.4)/acetonitrile mixtures, the diffusion coefficients are $D(L) = 3.20(4) \times 10^{-10}$ m$^2$ s$^{-1}$ and $D(CuL) = 3.02(4) \times 10^{-10}$ m$^2$ s$^{-1}$. This leads to $M(CuL) \approx 696(54)$ g mol$^{-1}$, which compares reasonably well with the value of 645 g mol$^{-1}$ calculated for CuL. This confirms that the species formed with excess or 1 equiv of ligand is the mononuclear complex CuL with $C_3$ symmetry in which the three sulfur atoms are coordinated to the Cu(I) ion. This complex is also evidenced by electrospray mass spectrometry (ES-MS), which shows an intense peak at $m/z$ 647 corresponding to $[CuLH_3]^+$. Intense dichroic bands develop in the CD spectrum of CuL with positive and negative maxima at (+) 209, (−) 238, (+) 262, and (−) 288 nm; the two latter bands are attributed to thiolate−Cu LMCT transitions.[12] The coordination geometry of Cu(I) in CuL was observed in the solid state with monodentate sulfur ligands providing mononuclear complexes with a $CuS_3$ geometry, which are known to co-oligomerize with excess Cu(I) to form $(Cu_2S_3)_x$ clusters.[14]

In excess Cu(I), all of the spectroscopic features changed dramatically, indicating the formation of a Cu(I) cluster. The LMCT transitions in the CD spectrum [(+) 259 and (−) 284 nm] were shifted to higher energy, and weak lower-energy bands with maxima at (−) 310 and (+) 340 nm appeared. These two latter bands were detected as tails in the absorption spectrum and are attributed to cluster-centered transitions.[12] Three isodichroic points were detected at 218, 252, and 325 nm in going from 1 to 2 equiv of Cu(I), demonstrating that the mononuclear complex CuL was transformed into only one cluster complex, $(Cu_2L)_x$. ES-MS spectra recorded with excess Cu(I) indicated the $x = 3$ cluster species $[Cu_6L_3H_5]^{2+}$ with the expected isotopic signature (Figure S3 in the SI). In contrast to CuL, this cluster exhibited broad resonances in its $^1$H NMR spectrum that may be characteristic of intramolecular dynamics associated with exchange of the sulfur ligands from one Cu ion to another in this polynuclear complex. The diffusion coefficient, $D[(Cu_2L)_x] = 2.0(1) \times 10^{-10}$ m$^2$ s$^{-1}$, confirmed the trimeric nature of this complex, giving $M[(Cu_2L)_x] \approx 2200(400)$ g mol$^{-1}$ and therefore $x = 3$. The polymetallic complex $Cu_6L_3$ can be associated with a $Cu_6S_9$ core, as described in some MTs, where each Cu(I) adopts a nearly trigonal planar coordination with exclusively bridging thiolates.[15]

The apparent stability constants of the two Cu(I) complexes at pH 7.4 were measured using UV−vis titrations in the presence of bathocuproine disulfonate (BCS), which forms an orange complex, $[Cu(bcs)_2]^{3-}$, of known stability.[16] The first Cu(I) ion is very tightly bound as demonstrated by the large value of $\beta_{11}$ for eq 1 [$\log\beta_{11} = 19.2(1)$]. The complexation of a second Cu(I) ion according to eq 2 is also very efficient [$\log K_2 = 20.7(3)$].

$$Cu(I) + L \rightleftarrows CuL \quad (1)$$

$$CuL + Cu(I) \rightleftarrows \frac{1}{3}Cu_6L_3 \quad (2)$$

The apparent affinity constant of the mononuclear complex CuL is significantly larger (by 1.8 orders of magnitude) than that of a model peptide of the copper chaperone Atx1, $P^C$, which affords only two cysteines (see Table 1).[8] The large stability of the CuL complex reflects the good complementarity of this tripodal ligand architecture, which provides three converging metal-binding thiolates and Cu(I) coordination.

To evaluate the selectivity of L for Cu(I), the complexes with Pb(II), Cd(II), Zn(II), and Hg(II) were studied as previously reported.[8] UV titrations demonstrated the formation of ML$^-$ complexes, which were also the only ions detected in the ES-MS spectra. The LMCT absorption bands were consistent with metal ions coordinated by three thiolates.[17] The apparent stability constants of the mononuclear complexes ML at pH 7.4, reported in Table 1, are also significantly enhanced in comparison to the corresponding $P^C$ complexes.[8] Furthermore, the selectivity for Cu(I) over Zn(II), a potential competitor in cells, is still very high ($\sim 10^9$).

***Table 1.*** Apparent Affinity Constants ($\log\beta_{11}^{app}$, where $\beta_{11}$=[ML]/[M][L]) for the Mononuclear Complexes of L and $P^C$ at pH 7.4 and 298 K

| | Ca(II) | Pb(II) | Zn(II) | Cd(II) | Cu(I) | Hg(II) |
|---|---|---|---|---|---|---|
| L | <4.2 | 10.1(1) | 10.3(2) | 11.8(3) | **19.2(1)** | >22.7 |
| $P^C$ [a] | – | 8.8(1) | 7.6(2) | 10.0(1) | **17.4(1)** | >19.4 |

[a] $P^C$ is a cyclodecapeptide modeling the binding loop of the copper chaperone Atx1: c(GMTCSGCSRP).[8]

In conclusion, we have developed a cysteine-based ligand that very efficiently chelates Cu(I) (the oxidation state present in cells) with a higher affinity than the MxCxxC sequence found in Cu transporters. This chelator is based on a tripodal pseudopeptide scaffold extended by three converging cysteines and mimics the selectivity of metallothioneins and other cysteine-rich proteins for the soft cations Cu(I) and Hg(II) over other divalent ions. This ligand providing three soft sulfur donors has a very high affinity for Cu(I) in either a mononuclear complex or the $Cu_6L_3$ cluster.

**Acknowledgment.** Financial support from the Cluster de recherche Chimie de la Région Rhône-Alpes is duly acknowledged. We thank Grégory Baral-Baron and Yves Chenavier for performing some of the titration experiments.

**Supporting Information Available:** Synthesis of L, physicochemical procedures, and NMR titration and ES-MS data. This material is available free of charge via the Internet at http://pubs.acs.org.

## References

(1) Andersen, O. *Chem. Rev.* **1999**, *99*, 2683.
(2) Rosenzweig, A. C. *Acc. Chem. Res.* **2001**, *34*, 119. Puig, S.; Thiele, D. J. *Curr. Opin. Chem. Biol.* **2002**, *6*, 171. Arnesano, F.; Banci, L.; Bertini, I.; Ciofi-Baffoni, S. *Eur. J. Inorg. Chem.* **2004**, 1583.
(3) Tao, T. Y.; Gitlin, J. A. *Hepatology* **2003**, *37*, 1241.
(4) Sarkar, B. *Chem. Rev.* **1999**, *99*, 2535.
(5) Bush, A. I. *Trends Neurosci.* **2003**, *26*, 207.
(6) Gaggelli, E.; Kozlowski, H.; Valensin, D.; Valensin, G. *Chem. Rev.* **2006**, *106*, 1995. Donnelly, P. S.; Xiao, Z. G.; Wedd, A. G. *Curr. Opin. Chem. Biol.* **2007**, *11*, 128.
(7) Rosenzweig, A. C.; O'Halloran, T. V. *Curr. Opin. Chem. Biol.* **2000**, *4*, 140. Opella, S. J.; DeSilva, T. M.; Veglia, G. *Curr. Opin. Struct. Biol.* **2002**, *6*, 217. Arnesano, F.; Banci, L.; Bertini, I.; Ciofi-Baffoni, S.; Molteni, E.; Huffman, D. L.; O'Halloran, T. V. *Genome Res.* **2002**, *12*, 255.
(8) Rousselot-Pailley, P.; Sénèque, O.; Lebrun, C.; Crouzy, S.; Boturyn, D.; Dumy, P.; Ferrand, M.; Delangle, P. *Inorg. Chem.* **2006**, *45*, 5510.
(9) Sénèque, O.; Crouzy, S.; Boturyn, D.; Dumy, P.; Ferrand, M.; Delangle, P. *Chem. Commun.* **2004**, 770.
(10) Koch, K. A.; Pena, M. M. O.; Thiele, D. J. *Chem. Biol.* **1997**, *4*, 549. Stillman, M. J. *Coord. Chem. Rev.* **1995**, *144*, 461.
(11) Gelinsky, M.; Vogler, R.; Vahrenkamp, H. *Inorg. Chem.* **2002**, *41*, 2560.
(12) Pountney, D. L.; Schauwecker, I.; Zarn, J.; Vasak, M. *Biochemistry* **1994**, *33*, 9699. Faller, P.; Vasak, M. *Biochemistry* **1997**, *36*, 13341.
(13) Waldeck, A. R.; Kuchel, P. W.; Lennon, A. J.; Chapman, B. E. *Prog. Nucl. Magn. Reson. Spectrosc.* **1997**, *30*, 39.
(14) Coucouvanis, D.; Murphy, C. N.; Kanodia, S. K. *Inorg. Chem.* **1980**, *19*, 2993. Zeevi, S.; Tshuva, E. Y. *Eur. J. Inorg. Chem.* **2007**, 5369.
(15) Nielson, K. B.; Atkin, C. L.; Winge, D. R. *J. Biol. Chem.* **1985**, *260*, 5342. Presta, A.; Green, A. R.; Zelazowski, A.; Stillman, M. J. *Eur. J. Biochem.* **1995**, *227*, 226.
(16) Xiao, Z.; Loughlin, F.; George, G. N.; Howlett, G. J.; Wedd, A. G. *J. Am. Chem. Soc.* **2004**, *126*, 3081.
(17) Matzapetakis, M.; Farrer, B. T.; Weng, T.-C.; Hemmingsen, L.; Penner-Hahn, J. E.; Pecoraro, V. L. *J. Am. Chem. Soc.* **2002**, *124*, 8042. Magyar, J. S.; Weng, T.-C.; Stern, C. M.; Dye, D. F.; Rous, B. W.; Payne, J. C.; Bridgewater, B.; Mijovilovich, A. M.; Parkin, G.; Zaleski, J. M.; Penner-Hahn, J. E.; Godwin, H. A. *J. Am. Chem. Soc.* **2005**, *127*, 9495.

JA901700A