J|A|C|S
COMMUNICATIONS

Published on Web 01/28/2003

Tetrathiomolybdate Causes Formation of Hepatic Copper−Molybdenum Clusters in an Animal Model of Wilson's Disease

Graham N. George,*,† Ingrid J. Pickering,† Hugh H. Harris,† Jürgen Gailer,‡ Dominik Klein,§ Josef Lichtmannegger,§ and Karl-Heinz Summer§

Stanford Synchrotron Radiation Laboratory, Stanford Linear Accelerator Center, 2575 Sand Hill Road, MS 69, Menlo Park, California 94025, and GSF National Research Center for Environment and Health, Institute for Ecological Chemistry and Institute for Toxicology, Ingolstädter Landstrasse 1, 85764 Neuherberg, Germany

Received October 23, 2002; E-mail: g.george@stanford.edu

Wilson's disease is a human illness in which large quantities of copper accumulate in various organs, including the brain and the liver. It is an autosomal recessive disease arising from a mutation in the ATP7B gene, which codes for a copper-transporting P-type ATPase involved in copper excretion.[1] If left untreated, it results in hepatitis, neurological complications, and death. Treatments such as dietary control (i.e., avoiding foods rich in copper), chelation therapy, and dietary supplementation with zinc ameliorate the effects of the disease, and patients with neurological symptoms have been successfully treated with tetrathiomolybdate.[2] Long-Evans Cinnamon (LEC) rats have a homologous mutation to Wilson's disease, which leads to fulminant hepatitis (acute liver failure) after about 90 days and ultimately results in death.[3] Both in Wilson's disease and in LEC rats, copper accumulates in lysosomes within hepatocytes.[4] Interperitoneal injection of aqueous solutions of tetrathiomolybdate $[MoS_4]^{2-}$ has been previously used to prevent or treat pathological symptoms in LEC rats.[5,6] Tetrathiomolybdate administered in this way is strikingly effective in treating LEC rats and can even reverse the effects of copper toxicity; it is also under investigation to suppress tumor growth and angiogenesis by inducing copper deficiency.[7] Microscopic examination of livers of treated LEC rats indicates that both copper and molybdenum are colocalized in lysosomes. We have investigated the molecular mechanism of tetrathiomolybdate as a potential treatment of Wilson's disease using X-ray absorption spectroscopy[8] at the Mo and Cu K-edges of isolated LEC rat liver lysosomes.[9−11]

Figure 1 shows the Cu and Mo K-edge extended X-ray absorption fine structure (EXAFS) of liver lysosomes from a tetrathiomolybdate-treated LEC rat, together with best fits and EXAFS Fourier transforms. Both data sets are dominated by backscattering from directly coordinated ligands, identified by curve-fitting as sulfur,[12] and show clear indications of longer-range metal−metal backscattering, indicating a multimetallic cluster. Quantitative curve-fitting analysis of the Mo data indicated 4 Mo−S at a distance of 2.24 Å, plus 3 Mo···Cu interactions at 2.70 Å, while the copper EXAFS indicated 3−3.5 Cu−S at 2.28 Å, with slightly less than one Cu···Mo at 2.70 Å. Thus, the EXAFS indicates that the metals have only sulfur ligands,[12] that each molybdenum has approximately three copper neighbors, while every copper has approximately one molybdenum neighbor.[13] In biological samples such as we have examined, the presence of some heterogeneity in the clusters is expected, and it therefore seems likely that a mixture of related species occurs within the lysosomes, possibly with different cluster nuclearities.

Chemical analysis[14] revealed a copper:molybdenum molar ratio of approximately 4.5:1, suggesting that approximately one-third of the lysosomal copper is not bound directly to molybdenum. An observed Cu···Mo coordination number of slightly less than 1 (see Supporting Information) may also reflect this. Thus, the extra Cu is not involved in a Cu−Mo cluster but must be coordinated by thiolate ligands as inclusion of nonthiolate ligands does not improve the fit.

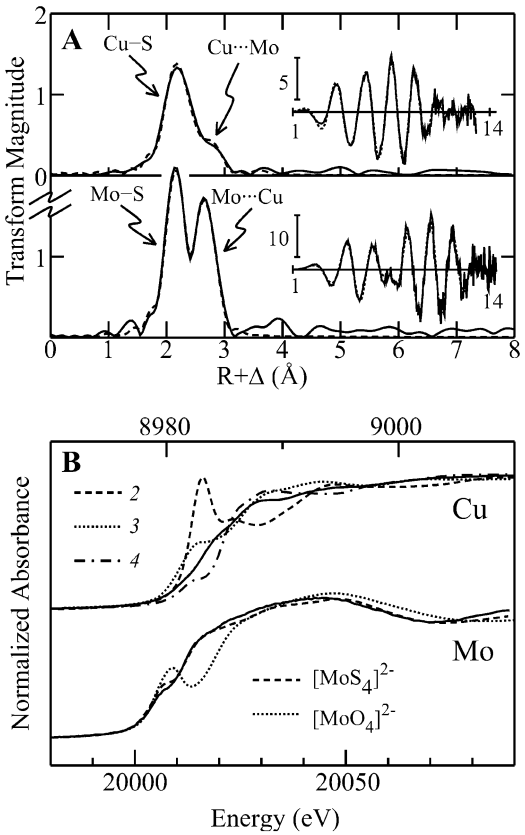

Figure 1. (A) Cu (upper) and Mo (lower) K-edge EXAFS Fourier transforms (phase-corrected for sulfur backscattering) and EXAFS (insets) of thiomolybdate-treated LEC rat liver lysosomes. The solid lines show experimental data, while the broken lines show the best fits. (B) Cu and Mo near-edge spectra (solid lines) as compared with the spectra of selected model compounds. For Cu, these are cuprous thiolates of different coordination numbers (as indicated): *2*, $[Cu(SC_{10}H_{12})_2]^-$; *3*, $[Cu_4(SPh)_6]^{2-}$; and *4*, *Desulfovibrio gigas* orange protein $[S_2MoS_2CuS_2MoS_2]^{3-}$.

The copper and molybdenum K near-edge spectra are shown in Figure 1B. The Mo spectrum strongly resembles that of tetrathiomolybdate (Figure 1B), suggesting thio coordinated Mo^{VI} with approximately tetrahedral geometry. The Cu K near-edge spectrum lacks the 8979 eV 1s → 3d peak characteristic of Cu^{II}, indicating a Cu^{I} oxidation state.[15] The spectra are unlike those of pure

† Stanford Synchrotron Radiation Laboratory.
‡ GSF Institute for Ecological Chemistry.
§ GSF Institute for Toxicology.

10.1021/ja029054u CCC: $25.00

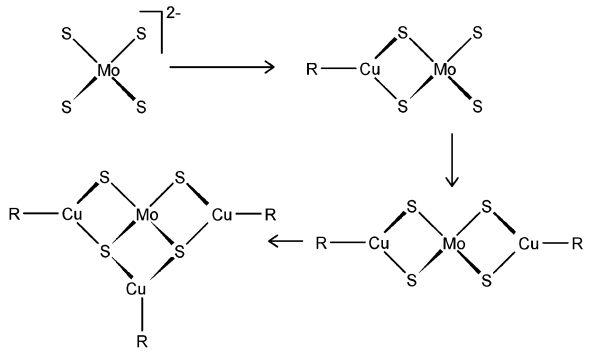

Figure 2. Schematic diagram of formation of polymetallic clusters by sequential addition of copper moieties to tetrathiomolybdate.

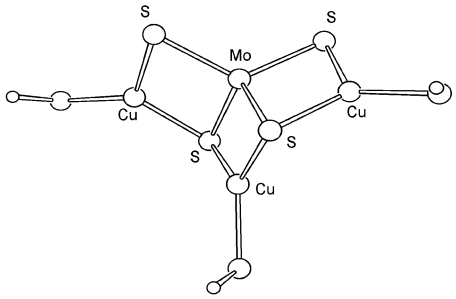

Figure 3. Computed structure of $[(HSCu)_3S_4Mo]^{2-}$.[18] The external ligands were approximated by HS^- groups.

standards but resemble a mixture of three-coordinate and four-coordinate species (Figure 1B).

The chemistry of copper−molybdenum−sulfur clusters has been well studied. Tetrathiomolybdate tends to form species bridged to copper by two of its sulfur ligands, with the copper coordination being completed by ligands external to the cluster (Figure 2). Tetrathiomolybdate can accommodate up to six copper atoms in this way, and structurally characterized examples are present in the Cambridge Structural Database[16] with one, two, three, four, and six coppers per molybdenum, three having the most entries. All show Mo···Cu interatomic distances close to 2.70 Å and Mo−S bond lengths close to 2.23 Å;[17] both of these distances are in excellent agreement with those observed in the tetrathiomolybdate-treated LEC rat samples and with those obtained by caclulation.[18] Cu−S bond lengths change systematically with Cu coordination number, from 2.17, 2.26, and 2.33 Å for two, three, and four coordination, respectively. Our observation of 2.28 Å is consistent with a mixture of species, having predominant trigonal with some four coordination, as was deduced from the near-edge. Together the data are consistent with the $[(RSCu)_3S_4Mo]^{2-}$ cluster, a possible structure of which is shown in Figure 3.[18] Additional long-range Cu···Cu interactions might be expected at about 3.63 and 5.25 Å. While small Fourier transform features appear at about these distances (Figure 1), these are too close to the intensity of the noise to be unambiguously observed. Simulations (not illustrated) indicate that these interactions should be weak, and additional damping is expected from the chemical heterogeneity of the sample.

Other possible structures such as the cubane of Hou et al.[19] can be ruled out because distinctly resolved Mo=S and Mo−S distances would be observed in the Mo K-edge EXAFS. A related biological Mo−Cu−S cluster has recently been observed in a protein of unknown function from the sulfate reducing bacterium *Desulfovibrio gigas*;[20] a binuclear cluster with a Mo−S−Cu core has very recently been reported for a bacterial CO dehydrogenase.[21] These are the only known biological mixed-metal sulfide clusters that do not contain iron.

In summary, the molecular basis for the beneficial effects of tetrathiomolybdate upon LEC rats appears to be the in vivo formation of copper−molybdenum−sulfur clusters, related to that shown in Figure 3. This in turn suggests that the mechanism of action may be a decrease in the bioavailability of the copper or a change in the redox properties (both resulting from cluster formation), either alone or in combination. These results have a wider implication for understanding the mechanism of other possible clinical uses of tetrathiomolybdate, such as in cancer therapy.[7]

Acknowledgment. SSRL is funded by the DOE, OBES, and OBER, and the NIH, NCRR BMTP. J.G. was supported by the Alexander von Humboldt Foundation.

Supporting Information Available: Tables of Mo and Cu K-edge EXAFS curve-fitting results (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Bull, P. C.; Thomas, G. R.; Rommens, J. M.; Forbes, J. R.; Cox, D. W. *Nat. Genet.* **1993**, *5*, 327−337.
(2) Brewer, G. J. *J. Trace Elem. Exp. Med.* **2000**, *13*, 51−61.
(3) Wu, J.; Forbes, J. R.; Chen, H. S.; Cox, D. W. *Nat. Genet*. **1994**, *7*, 541−545.
(4) The copper deposits are widely assumed to be in the form of copper metallothionein. Our preliminary XAS results indicate that this is not the case, and this will be the subject of future investigations.
(5) Suzuki, K. T.; Yamamoto, K.; Kanno, S.; Aoki, Y.; Takeichi, N. *Toxicology* **1993**, *83*, 149−158.
(6) Komatsu, Y.; Sadakata, I.; Ogra, Y.; Suzuki, K. T. *Chem.-Biol. Interact.* **2000**, *124*, 217−231.
(7) Pan, Q.; Kleer, C. G.; van Golden, K. L.; Irani, J.; Bottema, K. M.; Bias, C.; De Carvalho, M.; Mesri, E. A.; Robins, D. M.; Dick, R. D.; Brewer, G. J.; Merajver, S. D. *Cancer Res.* **2002**, *62*, 4854−4859.
(8) XAS measurements were carried out at the Stanford Synchrotron Radiation Laboratory (SSRL) as previously described [George, G. N.; Garrett, R. M.; Prince, R. C.; Rajagopalan, K. V. *J. Am. Chem. Soc.* **1996**, *118*, 8588−8592]. The extended X-ray absorption fine structure (EXAFS) oscillations χ(k) were quantitatively analyzed using EXAFSPAK [http://ssrl.slac.stanford.edu/exafspak.html].
(9) Klein, D.; Lichtmannegger, J.; Heinzmann, U.; Müller-Höcker, J.; Michaelsen, S.; Summer, K. H. *Eur. J. Clin. Invest.* **1998**, *28*, 302−310.
(10) Klein, D.; Lichtmannegger, J.; Heinzmann, U.; Summer, K. H. *J. Hepatol.* **2000**, *32*, 193−201.
(11) Female LEC rats were raised on a commercial rat diet (copper content: 11 mg kg^{-1}). At day 70, approximately 2 weeks before the onset of hepatitis, two animals were injected intraperitoneally with 5 mg of $(NH_4)_2MoS_4$/kg body weight twice a week (nine doses altogether). Fourteen days after the last treatment, at the age of 112 days, the experiment was terminated. The animals did not show any signs of liver disease, whereas untreated rats suffered from fulminant hepatitis from the age of about 90 days onwards. Hepatic heavy lysosomes were isolated as previously described[9,10] and prepared for XAS by mixing with glycerol (6:4, v/v).
(12) EXAFS cannot distinguish backscatters of similar atomic number; for example, while O and S can be readily distinguished, S and Cl are not, nor are N and O.
(13) Because of high mutual correlation with the Debye−Waller factor in the refinement, the accuracy of EXAFS-derived coordination numbers is only ±20%. In contrast, bond lengths are determined to an accuracy of better than ±0.02 Å.
(14) Elemental analysis used an inductively coupled plasma atomic emission spectrometer (Jobin Yvon JY66).
(15) Pickering, I. J.; George, G. N. *Inorg. Chem.* **1995**, *34*, 3142−3152.
(16) Allen, F. H.; Kennard, O. *Chem. Des. Autom. News* **1993**, *1*, 31−37
(17) Average values from relevant entries in the Cambridge Structural Database are given.
(18) Density functional theory calculations were carried out using the program Dmol[3] Materials Studio (Version 2.1) as previously described [Brown, K. R.; Keller, G. L.; Pickering, I. J.; Harris, H. H.; George, G. N.; Winge, D. R. *Biochemistry* **2002**, *41*, 6469−6476].
(19) Hou, H.; Liu, Y.; Xin, X.; Wu, Q.; Lu, S. *Synth. React. Inorg. Met.-Org. Chem.* **1995**, *25*, 1417−1421.
(20) George, G. N.; Pickering, I. J.; Yu, E. Y.; Prince, R. C.; Bursakov, S. A.; Gavel, O. Y.; Moura, I.; Moura, J. G. *J. Am. Chem. Soc.* **2000**, *122*, 8321−8322.
(21) Dobbeck, H.; Gremer, L.; Keifersauer, R.; Huber, R.; Meyer, O. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 15971−15976.

JA029054U