The Menkes Disease Protein Binds Copper via Novel 2-Coordinate Cu(I)−Cysteinates in the N-Terminal Domain

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Copper is an integral cofactor for numerous enzymes that play key roles in various cell processes, including oxidative metabolism, neurotransmitter and neuropeptide biosynthesis, free-radical detoxification, iron uptake, and maturation of connective tissue. Copper deficiency within the cells leads to cerebral degeneration, connective tissue and vascular defects, poor temperature control, and other pathologies. Menkes disease is the best known example of inborn copper deficiency in humans.[1] The disease phenotype is associated with mutations within the Menkes disease gene[2−4] that encodes a membrane protein with homology to the large family of cation-transporting P-type ATPases.[5] The Menkes disease protein (MNKp) has high sequence homology to the other human copper-transporting P-type ATPase, the Wilson's disease protein (WNDp),[6−8] mutations of which lead to vast depositions of copper in a number of tissues, primarily in liver, brain, and kidney. The molecular mechanism by which these two proteins maintain the fine-tuned balance of copper in the cell is still poorly understood.

The most prominent feature in the structures of MNKp and WNDp is the presence of a large cytoplasmic N-terminal domain composed of six repetitive metal-binding segments of ∼70 residues, each of which includes a conserved metal-binding motif, GMTCXXC, capable of binding a single Cu(I) ion.[9] This putative metal-binding sequence is also present in the human copper chaperone, HAH1, which has been implicated in the selective metalation of MNKp,[10,11] and in Ccc2 and Atx1, the yeast analogues on MNKp and HAH1, respectively.[12,13] It has thus been presumed that the metal coordination in all proteins that share the motif is essentially the same. NMR data on a Ag(I) derivative of the recombinant metal-binding repeat 4 (MBR4) of MNKp[14] suggested that the Ag(I) ion was linearly coordinated by the two Cys residues of the GMTCXXC motif, while XAS data on Hg(II) derivatives of Atx1[12] and the bacterial mercury transport protein MerP[15] also indicated 2-coordination. However, a recent EXAFS study of Atx1 reconstituted with its native metal, Cu(I), indicated three S ligands rather than the expected 2-coordination for Cu(I),[12] even though the NMR structural data on the related MBR4 placed the methionine ligand in a noncoordinating position, pointing away from the metal site. Here we communicate XAS results on the full-length N-terminal domain of the Menkes protein (N-MNKp), complexed with its native ligand, copper, which resolves this dilemma. The N-MNKp was metalated in vivo and then purified in its copper-bound form.[16] We demonstrate for the first time that the metal-binding repeats in the N-terminal domain of the Menkes disease protein bind Cu(I) in a novel 2-coordinate all-sulfur ligand environment.

The metal-binding repeats were occupied by Cu which was present in its reduced, Cu(I), form as evidenced by the lack of EPR signal at 77 K. EXAFS data[17] indicated an all-sulfur isostructural[18] environment for all Cu(I) atoms. The Fourier transform (Figure 1) exhibited a large peak at 2.1 Å with a minor shoulder at ∼2.5 Å (phase-corrected). The EXAFS (Figure 1, inset) could be well fit by two sulfur atoms at a distance of 2.16 ± 0.01 Å, a distance typical of 2-coordinate Cu(I)−S complexes.[19−22] Since the NMR data on the Ag(I) complex of MBR4 (see above) showed the presence of the conserved threonine and serine[23] of

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(1) Danks, D. M. In *The Metabolic Basis of Inherited Disease*; Scrives, C., Beaudet, A., Sly, W., Valle, D., Eds.; McGraw-Hill: New York, 1989; pp 1411−1432.

(2) Vulpe, C.; Levinson, B.; Whitney, S.; Packman, S.; Gitschier, J. *Nat. Genet.* **1993**, *3*, 7−13.

(3) Chelly, J.; Tumer, Z.; Tonnesen, T.; Petterson, A.; Ishikawa-Brush, Y.; Tommerup, N.; Horn, N.; Monaco, A. P. *Nat. Genet.* **1993**, *3*, 14−19.

(4) Mercer, J. F.; Livingston, J.; Hall, B.; Paynter, J. A.; Begy, C.; Chandrasekharappa, S.; Lockhart, P.; Grimes, A.; Bhave, M.; Siemieniak, D.; et al. *Nat. Genet.* **1993**, *3*, 20−25.

(5) Lutsenko, S.; Kaplan, J. H. *Biochemistry* **1995**, *34*, 15607−15613.

(6) Tanzi, R. E.; Petrukhin, K.; Chernov, I.; Pellequer, J. L.; Wasco, W.; Ross, B.; Romano, D. M.; Parano, E.; Pavone, L.; Brzustowicz, L. M.; Devoto, M.; Peppercorn, J.; Bush, A. I.; Sternlieb, I.; Evgrafov, O.; Piratsu, M.; Gusella, J. F.; Penchaszadeh, G. K.; Honig, B.; Edelman, I. S.; Soares, M. B.; Scheinberg, I. H.; Gilliam, T. C. *Nat. Genet.* **1993**, *5*, 344−350.

(7) Petrukhin, K.; Lutsenko, S.; Chernov, I.; Ross, B. M.; Kaplan, J. H.; Gilliam, T. C. *Hum. Mol. Genet.* **1994**, *3*, 1647−1656.

(8) Bull, P. C.; Thomas, G. R.; Rommens, J. M.; Forbes, J. R.; Cox, D. W. *Nat. Genet.* **1993**, *5*, 327−337.

(9) Lutsenko, S.; Petrukhin, K.; Cooper, M. J.; Gilliam, C. T.; Kaplan, J. H. *J. Biol. Chem.* **1997**, *272*, 18939−18944.

(10) Klomp, L. W.; Lin, S. J.; Yuan, D. S.; Klausner, R. D.; Culotta, V. C.; Gitlin, J. D. *J. Biol. Chem.* **1997**, *272*, 9221−9226.

(11) Hung, I. H.; Casareno, R. L.; Labesse, G.; Mathews, F. S.; Gitlin, J. D. *J. Biol. Chem.* **1998**, *273*, 1749−1754.

(12) Pufahl, R. A.; Singer, C. P.; Peariso, K. L.; Lin, S.; Schmidt, P. J.; Fahrni, C. J.; Culotta, V. C.; Penner-Hahn, J. E. *Science* **1997**, *278*, 853−856.

(13) Lin, S. J.; Pufahl, R. A.; Dancis, A.; O'Halloran, T. V.; Culotta, V. C. *J. Biol. Chem.* **1997**, *272*, 9215−9220.

(14) Gitschier, J.; Moffat, B.; Reilly, D.; Wood, W. I.; Fairbrother, W. J. *Nat. Struct. Biol.* **1998**, *5*, 47−54.

(15) Steele, R. A.; Opella, S. J. *Biochemistry* **1997**, *36*, 6885−6895.

(16) The N-MNKp domain was overexpressed in *Escherichia coli* as a fusion with maltose-binding protein (MBP) as described earlier.[9] The purity of MNKp was checked by SDS−PAGE and was found to be >80%. The total Cu concentration was measured by flame atomic absorption (Varian, AA-5), yielding 650 μM Cu.

(17) Cu K-edge (8.979 keV) extended X-ray absorption fine structure (EXAFS) and X-ray absorption near-edge structure (XANES) data were collected on beam line 7-3 at the Stanford Synchrotron Radiation Laboratory (40−100 mA, 3 GeV, Si[220], detuned 50% to reject harmonics). Data were collected in fluorescence mode using a high-count-rate Canberra 13-element Ge array detector with maximum count rates below 100 kHz. The samples (70 μL) were measured as aqueous glasses (>20% ethylene glycol) at 15 K. Data reduction was performed using the program EXAFSPAK (George, G. N., Stanford Synchrotron Radiation Laboratory, Menlo Park, CA, 1995). Energy calibration was achieved by reference to the first inflection point of a copper foil (8980.3 eV) which was simultaneously measured. Data analysis was carried out using the program EXCURV98 (Daresbury Laboratory, Warrington, U.K.) and the OPT module in EXAFSPAK with theoretical phase shifts and amplitude functions calculated by FEFF 7.0 (Zabinsky, S. I.; Rehr, J. J.; Ankudinov, A. L.; Albers, R. C.; Eller, M. J. *Phys. Rev. B* **1995**, *52*, 2995). Both programs gave equivalent results.

(18) EXAFS spectroscopy can only determine the average coordination environment of compounds that have multiple metal sites, but several factors suggest that the sites are isostructural. (i) The Cu−S distance of 2.16 Å is typical for linear 2-coordinate Cu−S.[19−22] (ii) The refined value of the Debye−Waller term of 0.0044 Å^2 for the Cu−S interaction is in the range expected for a single metal-binding site with homogeneous bond lengths. (iii) The intensity of the 8984-eV edge feature is close to that expected for linear 2-coordinate Cu−S bonds. Structural variation between the metal centers and, in particular, distortions toward 3-coordination would broaden the feature and significantly decrease its intensity.

(19) Bowmaker, G. A.; Clark, G. R.; Seadon, F. J. K.; Dance, I. G. *Polyhedron* **1984**, *3*, 535−544.

(20) Dance, I. G. *Aust. J. Chem.* **1978**, *31*, 2195−2206.

(21) Koch, S. A.; Fikar, R.; Millar, M.; O'Sullivan, T. *Inorg. Chem.* **1984**, *23*, 122−123.

(22) Fujisawa, K.; Imai, S.; Kitajima, N.; Moro-oka, Y. *Inorg. Chem.* **1998**, *37*, 168−169.

(23) Threonine and serine are conserved residues in five of the six repeats. Histidine replaces the threonine in repeat 3.

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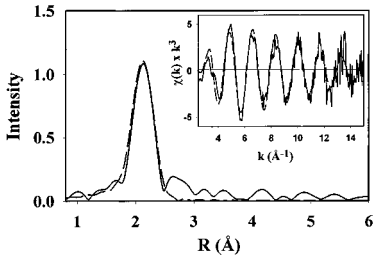

Figure 1. Experimental vs simulated Fourier transform and EXAFS (inset) for N-MNKp with multiple site occupancy ($n \geq 4$). The Fourier transform of the k^3-weighted EXAFS data was corrected for the Cu−S phase shift. Solid lines represent experimental data; dashed lines represent the best fit with two S at 2.16 Å ($\sigma^2 = 0.0044$ Å^2), EXCURV98 simulation.

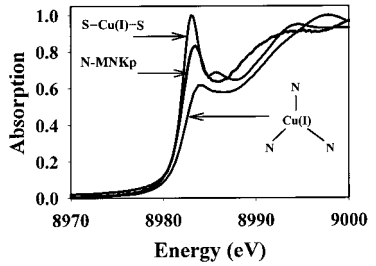

Figure 2. Cu K-edges for N-MNKp and model complexes: 2-coordinate model compound $[N(C_3H_7)_4][Cu(SC_{10}H_{13})_2]$ (thick);[21,30] Cu(I)−N-MNKp (medium); 3-coordinate model compound $[(Me_2Im)_3Cu(I)]^+$ (thin).[28]

the GMTCXSC motif within coordinating distance of the metal center,[14] we tested whether the weak shoulder to the high-R side of the main peak in the Fourier transform could be fit by an oxygen atom at a longer distance. Only minor improvements to the fit were obtained, and because of the low intensity of this feature, equally good fits could be obtained with a variety of scatterers.[24] Thus, while weak association of a third scatterer cannot be excluded, its identity is indeterminate.

These conclusions are further supported by edge data. Cu K-absorption edges for N-MNKp and two model complexes are shown in Figure 2. The data for N-MNKp reveal an intense peak at 8984 eV, assigned to the 4s → $4p_{x,y}$ transition in Cu(I) complexes.[25−27] Previous studies on model compounds have shown a correlation between the intensity of the 8984-eV peak and the coordination number and/or site symmetry of the Cu(I) center. Thus, 2-coordinate Cu(I) exhibits the most intense peak which is progressively attenuated with an increase in coordination number or distortion from planarity.[25−27] The intensity of the 8984-eV peak observed for N-MNKp is lower than that of a 2-coordinate model compound in which Cu is linearly coordinated by S[12,22] but is significantly more intense than what is observed for the 3-coordinate $[Cu(imid)_3]^+$.[28] This would be consistent with a small distortion from 2-coordination, perhaps via decreasing the S−Cu−S angle from 180°, or weak association of an additional scatterer at a longer distance.[24]

Our XAS results described here thus clearly demonstrate the existence of a biscysteinate Cu(I) structure in N-MNKp and, to our knowledge, provide the first example of such a structure in a metalloprotein. The results also differ notably from those of Cu(I)−Atx1 recently reported by Pufahl et al.[12] The latter study presented evidence for a 3-coordinate Cu(I) environment composed of two S scatterers at 2.25 Å and a more distant S at 2.40 Å. The authors concluded that a third coordination position was available for exogenous thiol coordination. They proposed a mechanism for copper transfer from Atx1 to Ccc2 involving a 3-coordinate associative intermediate, where the third S ligand was provided by a docked apo-Ccc2 molecule. While our results on the Cu(I) environment in N-MNKp are not inconsistent with this mechanism, they provide no supporting evidence for any 3-coordinate S-ligated intermediate. It is also clear that the mechanism of metal translocation from the cytosolic proteins to the membrane is not identical in yeast and mammalian cells. Unlike the human copper-transporting ATPases, the yeast copper-transporting ATPase Ccc2 has only two metal-binding repeats at the N-terminus, implying a simpler (probably 1:1) interaction between chaperone and ATPase. On the other hand, both of the human copper-transporting ATPases MNKp and WNDp have six metal-binding repeats, and site-directed mutagenesis of the conserved cysteine residues in the N-WNDp has revealed that at least three repeats must be preserved for function.[29] These data require a more complex translocation mechanism.

Two alternative mechanisms seem possible to explain the unidirectional transport of copper from the chaperone to the ATPase. If the copper-binding coordination in ATPase and chaperones is indeed identical, statistical arguments alone could explain why a multidomain structure is necessary for vectorial transport, since a Cu(I) ion [or copper chaperone loaded with Cu(I)] dissociating from the ATPase is n times more likely to reassociate with the apomultimer than with a monomeric apochaperone, where n is the number of domains present. The functional requirement for at least three domains would then suggest n must equal or exceed 3 for efficient vectorial copper transport. The alternative (more plausible) explanation presumes that the spatial arrangement of the subdomains is critical for function.[24] Further mechanistic interpretations must await structural information on the mode of interaction of the subdomains and the symmetry of subdomain packing.

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Supporting Information Available: A plot showing the EXCURV98 simulation with the Cu−Cu interaction included (1 page, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(24) Simulation of the outer shell feature by O, S, and Cu scatterers was tested using both EXAFSPAK and EXCURV98 simulation programs and gave the following results (R, σ^2, F): for EXAFSPAK: Cu−O, 2.56 Å, 0.004 Å^2, 0.419; Cu−S, 2.36 Å, 0.007 Å^2, 0.397; Cu−Cu, 2.60 Å, 0.013 Å^2, 0.391. For EXCURV98: Cu−O, 2.30 Å, 0.023 Å^2, 8.88; Cu−S, 2.72 Å, 0.010 Å^2, 8.32; Cu−Cu, 2.59 Å, 0.013 Å^2, 8.94. The goodness of fit parameter, F, is defined for each program as follows:

$$F_{\mathrm{EXAFSPAK}} = \sum_{i=1}^{n} k^6(\mathrm{data}_i - \mathrm{model}_i)^2 / \sum_{i=1}^{n} k^6(\mathrm{data}_i)^2;$$

$$F_{\mathrm{EXCURV98}} = \sum_{i=1}^{n} k^6(\mathrm{data}_i - \mathrm{model}_i)^2 / n \quad (1)$$

These results show that the only outer-shell EXAFS contribution that gives results consistent between both programs is a Cu−Cu interaction at 2.60 Å. This may suggest some interaction exists between individual copper binding domains of MNKp. A plot showing the EXCURV98 simulation with the Cu−Cu interaction included is provided as Figure S1 in the Supporting Information.

(25) Kau, L.-S.; Spira-Solomon, D. J.; Penner-Hahn, J. E.; Hodgson, K. O.; Solomon, E. I. *J. Am. Chem. Soc.* **1987**, *109*, 6433−6442.

(26) Pickering, I.; George, G. N.; Dameron, C. T.; Kurz, B.; Winge, D. R.; Dance, I. G. *J. Am. Chem. Soc.* **1993**, *115*, 9498−9505.

(27) Blackburn, N. J.; Strange, R. W.; Reedijk, J.; Volbeda, A.; Farooq, A.; Karlin, K. D.; Zubieta, J. *Inorg. Chem.* **1989**, *28*, 1349−1357.

(28) Sanyal, I.; Karlin, K. D.; Strange, R. W.; Blackburn, N. J. *J. Am. Chem. Soc.* **1993**, *115*, 11259−11270.

(29) Payne, A. S.; Gitlin, J. D. *J. Biol. Chem.* **1998**, *273*, 3765−3770.

(30) Data were kindly provided by J. E. Penner-Hahn, University of Michigan.