A Novel Protein-Bound Copper−Molybdenum Cluster

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Four categories of mixed-metal sulfide clusters are known to participate in enzyme catalysis; the nickel−iron−sulfur and nickel−iron−selenium−sulfur clusters of nickel hydrogenases[1] and the molybdenum−iron−sulfur and vanadium−iron−sulfur clusters of nitrogenases.[2] All of these clusters contain iron, and all are involved in redox reactions. We present herein X-ray absorption spectroscopic evidence for a previously unknown and quite different type of protein-bound mixed-metal sulfide cluster containing molybdenum and copper but no iron, isolated from the sulfate reducing organism *Desulfovibrio gigas*.

When *D. gigas* NCIB 9332 cells are grown at 37 °C in a lactate−sulfate medium under anaerobic conditions[3] an orange colored protein of as yet unknown function can be isolated. Chemical analyses[4] indicate that it contains copper and molybdenum in the ratio 1.0:2.1, with no other metal or metalloid present in significant amounts.

The orange protein Mo and Cu K-edge extended X-ray absorption fine structure (EXAFS)[5] spectra (Figure 1) clearly show both metal−sulfur and metal−metal backscattering. Quantitative curve-fitting analysis of the Mo data indicated 4 Mo−S at a distance of 2.21 Å, plus a single Mo···Cu interaction at 2.75 Å, while the copper EXAFS indicated 4 Cu−S at 2.31 Å, with two Cu···Mo at 2.74 Å. Thus, the EXAFS indicates that the metals have only sulfur ligands, and that each molybdenum has a single copper neighbor, while the copper has two molybdenum neighbors. The molybdenum K near-edge spectrum of the orange protein strongly resembles that of tetrathiomolybdate (Figure 2), suggesting thio-coordinated Mo^{VI} with approximately tetrahedral geometry, and the Cu K near-edge spectrum (Figure 2) lacks the 8979 eV 1s → 3d peak characteristic of Cu^{II},[9] indicating a Cu^{I} oxidation state. The protein contains negligible phosphorus[10] and chlorine, as deduced from examination of these K-edges (not illustrated).

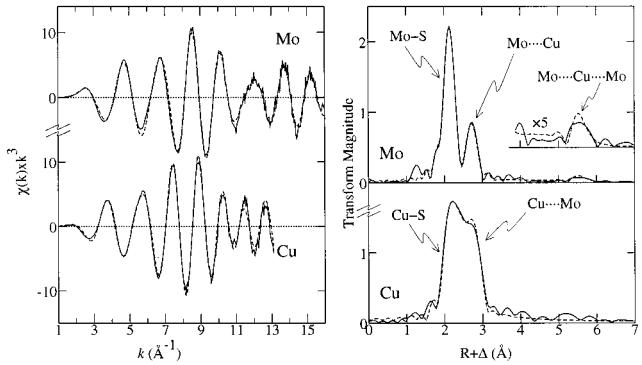

Figure 1. Mo K-edge and Cu K-edge EXAFS spectra of the orange protein (left), and corresponding Fourier transforms, phase-corrected for sulfur backscattering (right). The solid lines indicate experimental data, and broken lines, best fits: 4 Mo−S at 2.207(3) Å, with $\sigma^2 = 0.0038(3)$ Å^2, 1 Mo···Cu at 2.749(6) Å, with $\sigma^2 = 0.0027(3)$ Å^2, and 4 Cu−S at 2.305(6) Å, with $\sigma^2 = 0.0056(3)$ Å^2, 2 Cu···Mo at 2.736(6) Å, with $\sigma^2 = 0.0031(3)$ Å^2. σ^2 are the mean square deviations in interatomic distances, and the values in parentheses are 95% confidence limits (precisions) from the diagonal elements of the covariance matrix. We note that these will be an underestimate of the accuracies, which are typically better than ±0.02 Å for bond lengths. The inset shows the long-range interaction, tentatively identified as Mo···Cu···Mo with a Mo···Mo distance of 5.56(9) Å. The fit includes single-scattering ($\sigma^2 = 0.0055(21)$), plus three- and four-leg multiple scattering paths involving the metals ($\sigma^2 = 0.0064(27)$) and a Mo···Cu···Mo bond angle of 170°. The copper EXAFS data are truncated at $k = 13$ Å^{-1} because of traces of zinc in the preparation (the Zn K-edge occurs at this position).

The molybdenum L_{II}, L_{III}, and sulfur K near-edge spectra (Figure 2) provide direct information about the electronic structure of the cluster. The intense Mo L preedge absorption is due to dipole-allowed 2p → 4d transitions.[11] The two peaks which comprise this feature (see Figure 2, second derivatives) are due to 2p → 4d(t) and 2p → 4d(e) transitions, and their separation thus gives an excited-state Mo ligand-field splitting of about 1.34 and 1.24 eV for the L_{III} and L_{II} edges, respectively.[12] Because of the presence of a core-hole the observed (excited-state) ligand-field splitting will be slightly larger than that of the ground-state,

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(4) Purification was performed aerobically at pH 7.6, employing ion exchange DEAE-52 cellulose, hydroxyapatite (HTP), and Superdex 75 gel filtration. SDS-PAGE of the pure protein showed a single dye-stained band with a mobility corresponding to ~12 kDa, and gel filtration indicated that it is a monomer. One single N-terminal sequence was found for protein, confirming the purity. Electrospray mass spectrometry reveals a single peak corresponding to 11.9 kDa, agreeing with the calculated value from the sequence of 11.8 kDa. Metal analysis was performed by inductively coupled plasma emission analysis. (Bursakov, S. A.; Gavel, O. Y.; Moura, I.; Moura J. J. G., unpublished).

(5) X-ray absorption data acquisition was carried out at SSRL as previously described using beamline 7−3 for Cu and Mo K-edges,[6] and beamline 6−2 for S K and Mo L-edges.[7] The EXAFSPAK programs [http://ssrl.slac.stanford.edu/exafspak.html] were used to analyze the data, EXAFS curve-fitting employed ab initio phase and amplitude functions generated with the program FEFF v8.03.[8]

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(10) The lack of phosphorus indicates that the orange protein does not contain the molybdopterin cofactor [Johnson, J. L.; Rajagopalan, K. V. *J. Biol. Chem.* **1977**, *252*, 2017−2025].

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(12) Accurate positions for spectral features were estimated by pseudo-Voigt deconvolution using the program EDG_FIT.[9] We note that under idealized symmetry the L_{III} and L_{II} edges will exhibit differences in the transition probability to the various components of the 4d-manifold [e.g., (a) Sham, T. K. *J. Am. Chem. Soc.* **1983**, *8*, 2269−2273. (b) Tyson, T. A.; Case, D. A.; Hedman, B.; Hodgson, K. O. In *X-ray Synchrotron Radiation Research*; Balerna, A., Bernieri, E., Mobilio, S., Eds.; SIF: Bologna, 1990; pp 247−250].

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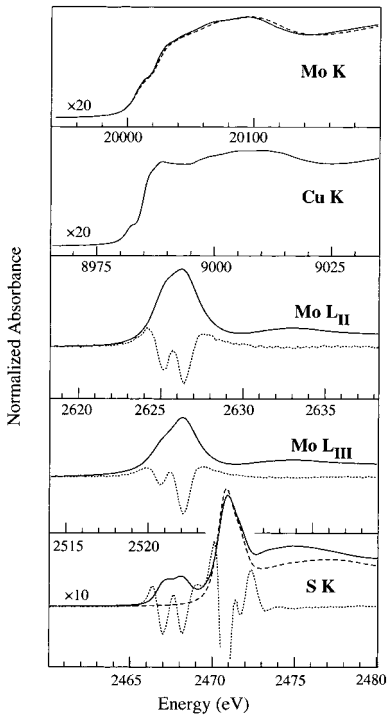

Figure 2. Mo K-edge, Cu K-edge, Mo L-edge, and S K-edge spectra of the orange protein. The broken line (- - -) in the Mo K-edge and S K-edge plots show the spectra of tetrathiomolybdate and methionine, respectively. The dotted lines (···) in the molybdenum L-edge and sulfur K-edge plots show the second derivative spectra. The Mo L-edge and S K-edge plots share the same energy range (20 eV) but have been shifted so as to align the transitions involving the Mo 4d-manifold. The relative ordinate scales of all plots have been adjusted for clarity.

which we estimate to be 1.19 eV.[13] The S K near-edge (Figure 2) shows clear features attributable to metal-bound sulfur; the double peak at 2467.6 eV is due to transitions to vacant orbitals with substantial sulfur 3p and metal d-orbital character. As Cu^{I} is formally 3d,[10] these must be molybdenum-based orbitals, and the splitting of this feature thus also gives an approximate Mo ligand-field splitting of about 1.09 eV, in reasonable agreement with the Mo L-edge value. The electronic spectrum (see Supporting Information) shows protein absorbance at 274 nm plus two bands at 480 and 338 nm ($\epsilon_{480} = 5500$ and $\epsilon_{338} = 10\,700$ M^{-1} cm^{-1}) and a weaker shoulder at 433 nm. The 480 and 338 nm bands can be assigned as ligand to metal charge-transfers involving Mo, and corresponding to a ligand-field splitting of 1.08 eV, in excellent agreement with the values derived from the X-ray spectra. The S K-edge transition energy of 2467.6 eV is lower than typically observed for thiolate ligands,[14] but characteristic of Mo bound sulfide (cf. $[MoS_4]^{2-}$ at 2467.3, 1.12 eV splitting[14]). The spectrum also shows features attributable to methionine but not cysteine;[15] in agreement, the amino acid sequence indicates no cysteine and three methionines.[16] The lack of cysteine suggests the cluster is not covalently tethered to the protein by an amino acid side chain (e.g., ref 17), except perhaps via methionine, although longer metal−sulfur bonds might be expected.

Together, the data are consistent with the $[S_2MoS_2CuS_2MoS_2]^{3-}$ cluster shown in Figure 3. This is expected to be EPR silent, as observed. The synthetic chemistry of Cu and Mo sulfide clusters has been well investigated, including species related to the orange protein cluster.[18,19] The cluster $[S_2MoS_2AgS_2MoS_2]^{3-}$ has Mo−S and Mo···Ag distances of 2.18 and 2.94 Å, respectively,[18] and $[S_2MoS_2CuSR]^{2-}$ has Mo−S, Cu−S, and Mo···Cu distances of

(13) Using the DGauss density functional theory we calculate that the effects of a core hole will be to increase the (observed) 4d-orbital splittings by approximately 8% relative to those of the ground state.

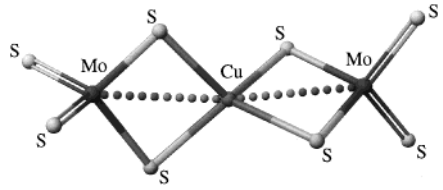

Figure 3. Postulated structure for the $[S_2MoS_2CuS_2MoS_2]^{3-}$ cluster of the orange protein.

2.19, 2.20, and 2.64 Å, respectively.[19] For the former, the increased metal−metal distances is primarily due to longer Ag−S bonds (2.52 Å), compared with Cu−S. The Cambridge Structural Database[20] suggests a typical four-coordinate Cu^{I}−S bond-length of about 2.30 Å, and the Cu−S distance observed with the orange protein is in excellent agreement with this value. Long distance Mo···Cu···Mo multiple scattering EXAFS would be expected for a linear arrangement of atoms within the cluster,[21] giving rise to a small Mo EXAFS Fourier transform peak at about 5.5 Å. Such a feature is indeed observed at marginally above the noise level of the data, which fits well to a Mo···Mo distance of 5.56(9) Å (Figure 1), although rather less intense than expected for a totally linear arrangement of metals. We attribute this to an approximately 10° deviation from linearity,[22] which has the effect of reducing the intensity of the multiple scattering interactions. A possible structure for the orange protein mixed-metal cluster is shown in Figure 3.[23]

In summary, we have structurally characterized a hitherto unknown protein-bound mixed-metal cluster in the orange protein of *D. gigas*. We deduce that it is the approximately linear species $[S_2MoS_2CuS_2MoS_2]^{3-}$. While the function of the orange protein is at present unknown, the $[S_2MoS_2CuS_2MoS_2]^{3-}$ cluster can potentially accept electrons, and a redox role seems quite plausible.

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Supporting Information Available: The electronic spectrum of the orange protein (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(16) Analysis of the intensity of the features attributable to methionine in the S K-edge data[15] indicated the occupancy of the cluster must be low (∼20−25%), with the presence of substantial apo-protein. Subsequent determination of the protein by amino acid composition confirmed that the copper−molybdenum cluster is present in approximately 20% of the protein molecules. The protein as-isolated was air stable and did not lose cluster with time.
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(22) Interestingly, the $[S_2MoS_2AgS_2MoS_2]^{3-}$ cluster reported by Muller et al.[18] also shows a 10° deviation from linearity.
(23) The EXAFS-derived interatomic distances can also be used to compute additional metrical information about the cluster. If all the Mo−S bond lengths were identical, the Mo−S−Cu bond angles would be 74.6°; as bridging sulfur is expected to have slightly longer bond lengths than the terminal sulfur, this value is a maximum. Maximum values for S−Mo−S and S−Cu−S bond lengths can also be estimated as 108.7° and 102.1°, respectively.