Observation of Ligand-Based Redox Chemistry at the Active Site of a Molybdenum Enzyme

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The mononuclear molybdenum enzymes all possess one or two molybdopterin cofactors coordinated to the molybdenum through the ditholene motif.[1] Despite this common feature, they exhibit quite diverse functionality.[1] The molybdenum enzymes previously have been described as all involving two-electron redox chemistry at molybdenum, coupled with the transfer of an oxygen atom from water via molybdenum to substrate, or the reverse.[1] While these rules still appear to hold for most molybdenum enzymes, and for their close relatives the tungsten enzymes,[2] it now seems that there are at least some exceptions. The recently discovered tungsten enzyme acetylene hydratase catalyzes a net hydration reaction, rather than a redox one.[3] Very recently it has been shown that formate oxidation to CO_2 by *Eschericia coli* formate dehydrogenase H (FDH_H) does not involve oxygen atom transfer.[4] This enzyme has also been shown to possess a potentially redox-active selenosulfide ligand to molybdenum,[5] with the selenosulfide sulfur probably being one of the sulfurs of the cofactor dithiolene. We present herein an extended X-ray absorption fine structure (EXAFS) spectroscopic study of the molybdenum site of *Desulfovibrio desulfuricans* ATCC 27774 formate dehydrogenase (FDH)[6] and show that under reducing conditions the selenosulfide group can be reduced. This is the first observation of ligand-based redox chemistry in a molybdenum enzyme.[7]

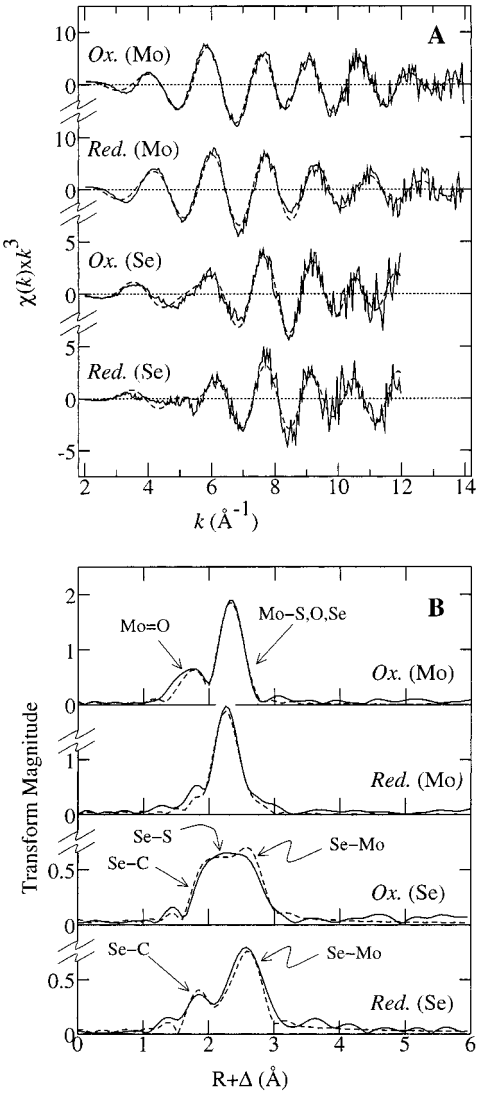

Figure 1. A shows the EXAFS spectra (solid lines) with best-fits (broken lines), and B shows the corresponding EXAFS Fourier transforms of *D. desulfuricans* FDH. From top to bottom, the figure shows the Mo K-edge data of oxidized enzyme, the Mo K-edge data of reduced enzyme, the Se K-edge data of oxidized enzyme, and the Se K-edge data of reduced enzyme.

Figure 1 shows the molybdenum and selenium K-edge EXAFS spectra[8] of *D. desulfuricans* FDH, together with the best fits[11] and the corresponding EXAFS Fourier transforms. The Mo K-edge data of the oxidized enzyme clearly indicate a mono-oxo active site (Mo═O at 1.70 Å), with approximately four Mo−S

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(7) EPR-active molybdopterin radicals (thought to be a Mo^{VI} trihydropterin species) have recently been detected in bacterial aldehyde dehydrogenases [Luykx, D. M. A.; Duine, J. A.; de Vries, S. *Biochemistry* **1998**, *37*, 11366−11375]. Although such species certainly indicate nonmetal-based redox chemistry, direct involvement of molybdenum ligands is not indicated.

(8) X-ray absorption spectroscopic data were collected on SSRL beamline 7-3 as previously described.[5,9] EXAFS oscillations $\chi(k)$ were quantitatively analyzed with EXAFSPAK [http://ssrl.slac.stanford.edu/exafspak.html] using ab initio phase and amplitude functions generated with *Feff* V7.02.[10] No smoothing, filtering, or related manipulation was performed upon the data. Enzyme samples, at approximately 0.5 mM Mo, were prepared as previously described[6] in 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid buffer at pH 7.5 and frozen in 3 × 10 × 10 mm Lucite sample cuvettes. The enzyme was reduced with 10 mM sodium dithionite solution in the presence of 40 μM methyl viologen for 4 min. Dithionite was chosen in preference to formate as reductant because the latter gives rise to a mixture of oxidation states (analysis of EXAFS of mixtures can be problematic). Data were collected at a sample temperature of 10 K and 16 35-min sweeps were averaged for each sample.

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(11) The best fits obtained from EXAFS curve-fitting were as follows: (a) Oxidized enzyme Mo K-edge, 4 Mo−S at 2.413(5) Å, with $\sigma^2 = 0.0049(3)$ Å^2, 1 Mo−Se at 2.570(17) Å, $\sigma^2 = 0.0057(15)$ Å^2 and 1 Mo═O at 1.704(5) Å, $\sigma^2 = 0.0023(5)$ Å^2. (b) Reduced enzyme Mo K-edge, 4 Mo−S at 2.367(5) Å, with $\sigma^2 = 0.0049(3)$ Å^2, 1 Mo−Se at 2.573(21) Å, $\sigma^2 = 0.0048(15)$ Å^2 and 1 Mo−O at 2.194(18) Å, $\sigma^2 = 0.0025(16)$ Å^2. (c) Oxidized enzyme Se K-edge, 1 Se−Mo at 2.574(8) Å, with $\sigma^2 = 0.0045(3)$ Å^2, 1 Se−S at 2.151(17) Å, $\sigma^2 = 0.0055(6)$ Å^2 and 1 Se−C at 2.050(10) Å, $\sigma^2 = 0.0020(9)$ Å^2. (d) Reduced enzyme Se K-edge, 1 Se−Mo at 2.569(3) Å, with $\sigma^2 = 0.0058(3)$ Å^2 and 1 Se−C at 1.968(8) Å, $\sigma^2 = 0.0029(8)$ Å^2. σ^2 are the Debye−Waller factors, and the values in parentheses are the estimated standard deviations (precisions) obtained from the diagonal elements of the covariance matrix.

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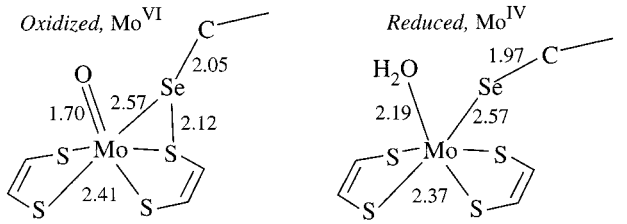

Figure 2. Postulated structures for the active site of oxidized and reduced *D. desulfuricans* FDH. Note that geometry is not directly available from EXAFS and that this is thus somewhat tentatively proposed from the geometries observed crystallographically for related enzymes.[13] The distances shown are those determined from the EXAFS curve-fitting analysis shown in Figure 1.

ligands at 2.42 Å and a single Mo−Se ligand at 2.57 Å. The Se K-edge EXAFS data indicate the presence of a Se−Mo at 2.57 Å (in excellent agreement with the Mo K-edge data), a Se−C at ∼2.0 Å, and a Se−S interaction at 2.15 Å.[12] The presence of a selenosulfide ligand has previously been demonstrated for *E. coli* FDH_H,[5] with the selenosulfide sulfur most likely originating from one of the cofactor dithiolene ligands.[5,13] A similar ligand configuration for oxidized *D. desulfuricans* FDH is proposed in Figure 2. The Mo K-edge data of the reduced enzyme indicate a des-oxo site, with one long Mo−O at 2.19 Å, approximately four Mo−S ligands at 2.38 Å, and one Mo−Se at 2.57 Å. The selenium K-edge data of reduced enzyme also indicate one Se−Mo at 2.57 Å, and one Se−C at ∼1.9 Å, but in contrast with the oxidized protein no Se−S interaction was required to fit the data. Comparison of the Se K-edge EXAFS Fourier transforms shown in Figure 1B clearly illustrate the absence of a Se−S interaction in the reduced sample.

The Mo K-edge results are consistent with a mono-oxo Mo^{VI} site for the oxidized *D. desulfuricans* FDH and a des-oxo Mo^{IV} site for the reduced enzyme. A similar active site chemistry is well established with other molybdenum enzymes,[1,9] with the Mo═O ligand being protonated to yield Mo−OH or $Mo-OH_2$ in reduced active sites. The fact that the molybdenum and the selenosulfide have both apparently been reduced suggests that the active site has undergone a total four-electron reduction. Such a multi-redox step may be the result of the vigorous conditions used for reduction, but the finding that the selenosulfide ligand is reducible has important implications for the catalytic mechanism. It is possible that in formate dehydrogenases the selenosulfide ligand to molybdenum, rather than the metal itself, performs the redox-active role during catalysis in this novel group of enzymes. This hypothesis is reinforced by the data from *E. coli* FDH_H. Both X-ray crystallography[13] and X-ray absorption spectroscopy[5] suggest des-oxo active site structures for oxidized *E. coli* FDH_H with the molybdenum possessing a Mo−OH or $Mo-OH_2$ ligand. Such structures would be quite unprecedented for a putative Mo^{VI} complex and should thus be considered as chemically improbable. Furthermore, the EXAFS study[5] indicated almost identical structures for the Mo sites of oxidized and dithionite-treated *E. coli* FDH_H, suggesting similar molybdenum oxidation states.[5,14] It now seems probable that oxidized *E. coli* FDH_H contains a formal Mo^{IV} site and that the catalytic two-electron redox reaction converting formate to CO_2 instead reduces the selenosulfide ligand, with the molybdenum filling a more passive electrochemical role. The potential importance of sulfur-based ligand redox chemistry for molybdenum enzymes has recently been discussed by Stiefel.[15]

Finally, we will discuss the molybdenum EPR spectroscopy of these enzymes. EPR signals attributed to Mo^V have been observed by addition of formate to *E. coli* FDH_H,[16] and two different types of signal have been observed in *D. desulfuricans* FDH by using redox potentiometry and by the addition of formate.[6] Although the formate-induced spectra from both enzymes are quite distinct, they both possess proton hyperfine couplings, and in the case of the *E. coli* enzyme this coupled proton has been shown to originate from the α-proton of formate.[16] For *D. desulfuricans* FDH, Mo^V species could in principle be generated either by one-electron reduction of the Mo^{VI} site or by total three-electron reduction of both the selenosulfide ligand and Mo^{VI}. For *E. coli* FDH_H, the presence of selenium coordination in the signal-giving species has been definitively shown by observation of large ^{77}Se hyperfine couplings in isotopically enriched enzyme,[16] indicating extensive spin delocalization onto selenium.[4,5] One-electron reduction of a Mo^{IV} species (see discussion above) would normally be expected to yield Mo^{III}. Although there are very few reports in the literature of well-characterized mononuclear Mo^{III} compounds with thiolate ligands,[17] it is expected that such species would be low-spin $S = {}^1/_2$, would have somewhat slightly higher *g*-values and anisotropy (when compared to Mo^V), and slightly lower Mo hyperfine couplings. Given the importance of ligand effects in Mo^V EPR spectra[18] we thus expect these two oxidation states to be difficult to distinguish by EPR alone, and it seems possible that the reported *E. coli* FDH_H Mo^V EPR signals are in fact from Mo^{III}.[19] In any case, the molybdenum EPR spectroscopy of formate dehydrogenases is clearly far from being well understood, and future X-ray absorption spectroscopic studies of EPR-active species may provide clarification.

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(12) We note that EXAFS cannot readily distinguish between backscatterers of similar atomic number. For example, although Se−C and Se−S EXAFS are trivial to tell apart, Se−S and Se−Cl EXAFS are not. It is therefore possible that the selenosulfide sulfur observed in the Se EXAFS is in fact a chloride. We consider this possibility unlikely, as the samples were prepared in chloride-free buffer.

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(14) We note that in this case the selenosulfide was retained in the dithionite-treated enzyme, although conditions of reduction differed in that no methyl viologen was added.

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(17) Reports of EPR studies of low-spin Mo^{III} complexes with thiolate ligands are restricted to solution species [e.g., Mitchell P. C. H.; Scarle, R. D. 1973, Proceedings of the Climax Molybdenum Meeting]. There are, however, reports of low-spin Mo^{III} EPR studies of well-characterized complexes with ligands that are not biologically relevant [e.g., Pleune, B.; Poli, R.; Fettinger, J. C. *J. Am. Chem. Soc.* **1998**, *120*, 3257−3258].

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(19) It is also possible that induced-redox chemistry might play a role in the generation of EPR active-species [e.g., see: Coyle, C. L.; Harmer, M. A.; George, G. N.; Stiefel, E. I. *Inorg. Chem.* **1990**, *29*, 14−19]. For example, a one-electron reduction of a Mo^{IV} active site, coupled with an induced-redox reaction in which the selenosulfide was reduced, would yield Mo^V.