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X-ray Absorption Spectroscopy of Selenate Reductase

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The metal sites of selenate reductase from *Thauera selenatis* have been characterized by Mo, Se, and Fe K-edge X-ray absorption spectroscopy. The Mo site of the oxidized enzyme has 3 to 4 sulfur ligands at 2.33 Å from two molybdopterin cofactors, one Mo═O group at 1.68 Å and one Mo–O with an intermediate bond length of 1.81 Å. The reduced enzyme has a des-oxo active site, again with about four Mo–S ligands (at 2.32 Å) and possibly one oxygen ligand at 2.22 Å. The enzyme was found to contain Se in a reduced form (probably organic) although the sequence does not indicate the presence of selenocysteine. The Se is coordinated to both a metal (probably Fe) and a lighter scatterer such as carbon.

The mononuclear molybdenum enzymes all contain one or two molybdopterin dithiolene cofactors coordinated to the metal, yet exhibit remarkable functional diversity.[1,2] The majority of molybdenum enzymes that have been described to date catalyze two-electron redox reactions involving Mo^{IV} and Mo^{VI},[1,2] coupled with the transfer of an oxygen atom from substrate via molybdenum to water or the reverse. The enzymes have been divided into three families based upon their oxidized active site structures.[2] (i) The xanthine oxidase family has a single molybdopterin dithiolene, with the molybdenum coordination completed by an oxo, a thio, and an $-OH_2$ or $-OH$ ligand. (ii) The sulfite oxidase family has one molybdopterin dithiolene, one cysteinyl sulfur, and two oxo ligands to molybdenum. (iii) The dimethyl sulfoxide (DMSO) reductase family has two molybdopterin dithiolene ligands, one oxo, and one amino acid ligand (e.g. Ser, Cys, SeCys, etc.).[3−5] The enzyme arsenite oxidase from *Alcaligenes faecalis* has been recently characterized by X-ray crystallography[6] and X-ray absorption spectroscopy.[7] This enzyme perhaps represents a fourth family as it lacks a coordinated amino acid residue, and has four sulfur donors (from two molybdopterin dithiolenes), one Mo═O at 1.70 Å and one unusually short Mo−O, possibly a Mo−OH, at 1.83 Å. It has been argued[7] that this active site resembles the tungsten enzymes.[8]

The selenate reductase from *Thauera selenatis*[9,10] is a soluble periplasmic molybdenum enzyme that catalyzes the two electron reduction of selenate to selenite:

$$SeO_4^{2-} + 2H^+ + 2e^- \rightarrow SeO_3^{2-} + H_2O$$

The enzyme is a trimeric $\alpha\beta\gamma$ complex with a molecular weight of ∼160000 Da; it contains one molybdenum, approximately three iron−sulfur (probably Fe_4S_4) clusters, and a single cytochrome *b* prosthetic group per $\alpha\beta\gamma$ complex.[9] Sequence similarities with other molybdenum enzymes suggest that selenate reductase fits into a distinct clade within the DMSO reductase family, along with dimethyl sulfide dehydrogenase and ethyl benzene dehydrogenase.[11] Physiologically, the enzyme is the terminal reductase supporting anaerobic growth on acetate in the presence of selenate. It

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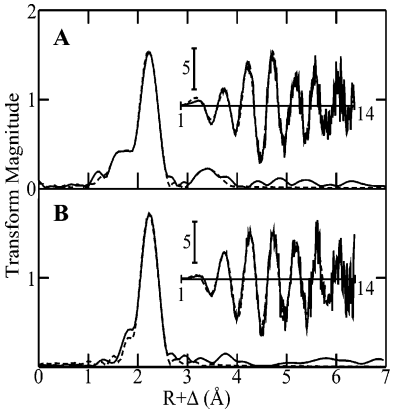

Figure 1. Fourier transforms (Mo−S phase-corrected) of the EXAFS spectra (shown in insets) of oxidized (A) and reduced (B) selenate reductase. The solid lines show the experimental data and the broken lines the best fits. Insets show the EXAFS spectra, k (Å^{-1}) on the abscissa and $\chi(k) \times k^3$ on the ordinate.

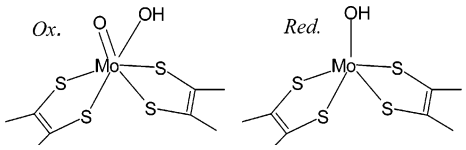

Figure 2. Postulated structures for the molybdenum active site of oxidized and reduced selenate reductase. Note that the number of sulfur ligands in the oxidized enzyme is somewhat ambiguous.[15]

is the only dissimilatory selenate reductase known, and is thus of considerable interest both in its own right and as a novel member of the molybdenum enzymes.

We describe herein a first study of the active site structure of *T. selenatis* selenate reductase using X-ray absorption spectroscopy.[12,13] Figure 1 shows the Mo K-edge extended X-ray absorption fine structure (EXAFS) and Fourier transforms, together with the results of a quantitative analysis.[14] Curve-fitting analysis of the oxidized enzyme indicates an active site with approximately four Mo−S ligands at 2.33 Å, one Mo═O at 1.68 Å, and one unusually short Mo−O at 1.81 Å. The difference between the two molybdenum oxygen bond lengths is close to the limit of resolution of our data,[3] but if the fit is restricted to a single molybdenum−oxygen bond length, a much poorer fit results with two oxygens at 1.73 Å and a chemically unreasonable Debye−Waller factor. Our conclusions are illustrated in Figure 2. The oxidized active site in some respects resembles that of oxidized *A. faecalis* arsenite oxidase.[7] This enzyme has two molybdopterin cofactors bound to Mo[6,7] and is unique among such enzymes in having no amino acid side chain ligand to Mo. Like selenate reductase, oxidized arsenite oxidase has one unusually short Mo−O bond at 1.83 Å plus a more normal Mo═O at 1.70 Å. One notable difference is that the Mo−S bond lengths of oxidized selenate reductase are unusually short (2.33 Å vs 2.47 Å in arsenite oxidase[7]). Bond-valence sum calculations[15] suggest that a formal oxidation state of Mo^{VI} would be satisfied with three sulfurs at 2.33 Å, and it is possible that the oxidized site has three Mo−S donors, and not four (despite slightly better fit-errors obtained with four). Analysis of the EXAFS of the reduced enzyme (Figure 1) indicates a des-oxo site, still with approximately four Mo−S ligands at 2.32 Å, plus a probable contribution from one or two Mo−O ligands at 2.22 Å (Figure 2B). The identification of the Mo−O ligands is, however only tentative due to correlation with the strong Mo−S contribution and the somewhat lower signal-to-noise of the reduced data set.[16] The similarity with arsenite oxidase might suggest that selenate reductase lacks an amino acid ligand. But enzymes with homology to selenate reductase do possess such a ligand (e.g. *Escherichia coli* NarG has an aspartate bound[17]). The oxidized enzyme also showed indications of long-range backscattering at around 3.4 Å. This might arise from the four pterin dithiolene carbon atoms, and the EXAFS of this interaction can be adequately modeled by using two different Mo···C interactions at 3.36 and 3.61 Å, respectively, but definitive identification of these long-range scatterers must await later studies on more concentrated enzyme.

One unexpected finding of our study was that the as-isolated enzyme contains selenium. Examination of the X-ray fluorescence spectra (not illustrated) indicated that Se is approximately equimolar to molybdenum. The Se K near-edge spectra (Figure 3A) clearly indicate that this Se is neither substrate (selenate) nor product (selenite), but instead is a more reduced form. Quantitative analysis of the Se K-edge EXAFS spectrum (Figure 3) indicates the presence of Se−C at 1.96 Å, plus a substantially heavier scatterer, possibly iron (but not Mo) at 2.41 Å. There is also some additional outer-shell structure evident (Figure 3), but despite accumulation of a large number of scans, our studies of the selenium coordination were limited by the noise content of the data.[16] Thus, although a combination of Se−C and Se−Fe gives the best fit,[18] it is difficult to distinguish between this and a three-component fit with Se−C at 1.98

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(13) Selenate reductase (∼0.1 mM Mo final concentration) was isolated as described by Schröder et al.[9,10] X-ray absorption data acquisition was carried out at SSRL as previously described using beamline 7-3.[3] The data were analyzed using the EXAFSPAK programs [http://ssrl.slac.stanford.edu/exafspak.html], and EXAFS curve-fitting employed ab initio phase and amplitude functions generated with the program FEFF v8.03.

(14) Best fits for oxidized enzyme were obtained with 4 Mo−S at 2.328(4) Å, σ^2 = 0.0052(2) Å^2, 1 Mo═O at 1.683(6) Å, σ^2 = 0.0018(9) Å^2, 1 Mo−O at 1.81(1) Å, σ^2 = 0.0029(13) Å^2, and for the reduced enzyme with 4 Mo−S at 2.322(8) Å, σ^2 = 0.0049(16) Å^2, ∼1 Mo−O at 2.22(8) Å, σ^2 = 0.0038(9) Å^2. The long-range interactions in the data of the oxidized sample could be modeled with two different Mo···C interactions at 3.36(1) Å and 3.61(1) Å, but, as discussed in the text, this assignment is not unambiguous. The values quoted in parentheses indicate the standard deviations (precisions) in the last digit(s) estimated from the diagonal elements of the covariance matrix.

(15) Bond valence sum calculations were carried out as previously described [George, G. N.; Hilton, J.; Rajagopalan, K. V. *J. Am. Chem. Soc.* **1996**, *118*, 1113−1117]. Four sulfur ligands give a bond-valence sum of 7.4, but if only three are used, then a value of 6.3 is obtained. While these results are not definitive, they do suggest that the sulfur coordination might be less than four.

(16) Because of the low concentration of the sample (∼0.1 mM) the data required the average of twenty-eight 32 min scans.

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(18) Best fits for the Se K-edge EXAFS of the oxidized enzyme were obtained with 1 Se−C at 1.962(8) Å, σ^2 = 0.0015(6) Å^2; 1 Se···Fe at 2.410(5) Å, σ^2 = 0.0029(2) Å^2. The long-distance interaction could be fitted with 1 Se···Fe at 4.04(1) Å, σ^2 = 0.0056(2) Å^2, but, as discussed in the text, this assignment is not unambiguous.

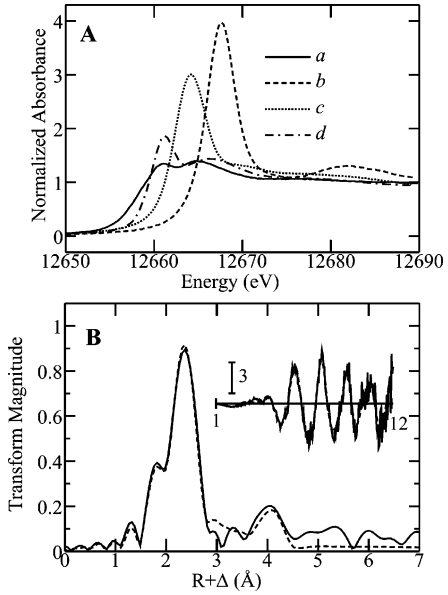

Figure 3. (A) Se K near-edge spectra of oxidized selenate reductase (a) compared with the solution spectra (at neutral pH) of selected selenium species: selenate (b), selenite (c), and selenomethionine (d). (B) Fourier transform (Se−Fe phase-corrected) of the Se EXAFS spectrum (shown in inset) of oxidized selenate reductase. The solid lines show the experimental data and the broken lines the best fit. The inset shows the EXAFS spectrum, k (Å^{-1}) on the abscissa and $\chi(k) \times k^3$ on the ordinate.

Å, Se−S at 2.18 Å and Se−Mo at 2.59 Å, similar to the coordination deduced for formate dehydrogenases.[19,20] Nevertheless, Se−Mo coordination seems unlikely, since a Se backscatterer at 2.59 Å would give rise to a well-resolved peak in the Mo EXAFS Fourier transform, which is not observed. Indeed, the fits to the Mo EXAFS deteriorated on including a Se neighbor (not illustrated). Our Se−Fe bond length of 2.41 Å compares reasonably well with that of 2.46 Å reported for the ferrous compound $[Fe(SePh)_4]^{2-}$.[21] The presence of Se−C would seem to suggest the presence of selenocysteine. In prokaryotes selenocysteine is coded for by the stop codon UGA, with a *cis*-acting mRNA element called the selenocysteine insertion sequence (SECIS), which can form a stem−loop structure.[22−24] *T. selenatis* selenate reductase does not contain the SECIS sequence,[10] and so the selenium is unlikely to be selenocysteine. Because Se−C EXAFS is essentially indistinguishable from Se−O and Se−N, other possibilities include posttranscriptional modification of a residue (perhaps a serine). Although the presence of some sort of modified iron−sulfur cluster seems quite

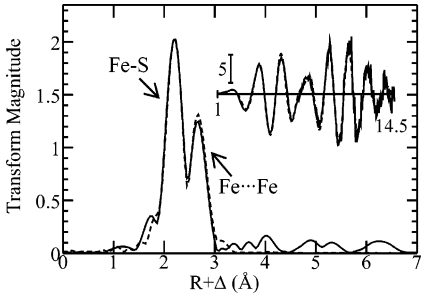

Figure 4. Fourier transform (Fe−S phase-corrected) of the Fe K-edge EXAFS spectrum (shown in inset) of oxidized selenate reductase. The solid lines show the experimental data and the broken lines the best fits. The inset shows the EXAFS spectrum, k (Å^{-1}) on the abscissa and $\chi(k) \times k^3$ on the ordinate.

plausible, the exact nature of the selenium site of selenate reductase remains uncertain, and resolution must await future spectroscopic and crystallographic studies.

The iron K-edge EXAFS spectra and Fourier transforms of oxidized selenate reductase are shown in Figure 4. The spectra are typical of iron−sulfur clusters, with 4 Fe−S at 2.27 Å and 2−3 Fe···Fe at 2.72 Å.[25] No evidence of Fe−N EXAFS from the cytochrome *b* was observed. However, this is expected as the iron−sulfur clusters contribute more than 90% of the total iron, and this, in combination with the inherently stronger EXAFS of iron−sulfur clusters, would effectively swamp any contribution from the cytochrome. Similarly, if the selenium is indeed coordinated to an iron (as we have discussed above), we do not expect to observe selenium backscattering in the iron EXAFS due to predominance of the Fe−S and Fe···Fe backscattering.

In summary, we have shown that selenate reductase contains an active site that is characteristic of the DMSO reductase family of molybdenum enzymes. The oxygen coordination of the active site may be similar to that of *A. faecalis* arsenite oxidase. The enzyme also contains a reduced selenium species that is associated with a metal ion, probably iron. The similarity to arsenite oxidase suggests the possibility that the molybdenum is not ligated by an amino acid.

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(25) Best fits for the Fe K-edge EXAFS of the oxidized enzyme were obtained with 4 Fe−S at 2.368(2) Å, $\sigma^2 = 0.0036(1)$ Å^2; 2 Fe···Fe at 2.716(2) Å, $\sigma^2 = 0.0032(1)$ Å^2.