Inorg. Chem. **2007**, *46*, 3097–3104

Interaction of Product Analogues with the Active Site of *Rhodobacter Sphaeroides* Dimethyl Sulfoxide Reductase

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Received October 5, 2006

We report a structural characterization using X-ray absorption spectroscopy of *Rhodobacter sphaeroides* dimethyl sulfoxide (DMSO) reductase reduced with trimethylarsine and show that this is structurally analogous to the physiologically relevant dimethyl sulfide reduced DMSO reductase. Our data unambiguously indicate that these species should be regarded as formal Mo^{IV} species and indicate a classical coordination complex of trimethylarsine oxide, with no special structural distortions. The similarity of the trimethylarsine and dimethyl sulfide complexes suggests, in turn, that the dimethyl sulfide reduced enzyme possesses a classical coordination of DMSO with no special elongation of the S–O bond, as previously suggested.

Introduction

The mononuclear molybdenum enzymes all possess one or two molybdopterin ditholene cofactors coordinated to the metal (Figure 1A) yet exhibit remarkably diverse functionality. Nevertheless, the majority that have been characterized to date catalyze two-electron-redox reactions coupled to the transfer of an O atom to or from water. During the catalytic cycle, the molybdenum cycles between Mo^{VI} and Mo^{IV} oxidation states. Hille[1] has divided the molybdenum enzymes into three families based on the active site structures of the prototypical enzymes of each family, i.e., the xanthine oxidase family, the sulfite oxidase family, and the dimethyl sulfoxide (DMSO) reductase family. DMSO reductase from *Rhodobacter sphaeroides*, together with the essentially identical *Rhodobacter capsulatus* enzyme, is considered the prototypical member of the DMSO reductase family of molybdenum enzymes. It catalyzes the reduction of DMSO to dimethyl sulfide:

$$\text{enzyme}-Mo^{IV} + (CH_3)_2S{=}O + 2H^+ \rightarrow \text{enzyme}-Mo^{VI} + (CH_3)_2S + H_2O$$

DMSO reductase has been extensively investigated by both spectroscopic and crystallographic studies. The active site structures of the *R. sphaeroides*[2] and *R. capsulatus*[3,4] enzymes were previously the subject of a controversy. Crystallographic studies by three different groups suggested dramatically different active site structures, within essentially identical polypeptide structures. All of these data were inconsistent with previous extended X-ray absorption fine structure (EXAFS) spectroscopy[5] and with subsequent resonance Raman spectroscopy.[6] Furthermore, a later EXAFS study[7] showed that the active site structures proposed from crystallography were all substantially chemically unreasonable. For example, atoms that were supposedly nonbonded had overlapping van der Waals radii, and structures contained

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10.1021/ic0619052 CCC: $37.00
Published on Web 03/16/2007

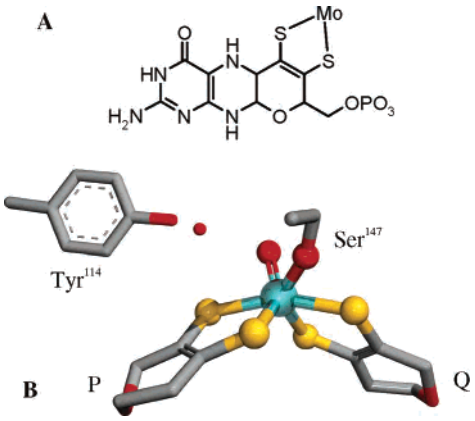

Figure 1. (A) Schematic structure for the molybdopterin molybdenum cofactor. (B) Crystal structure for the active site of oxidized (Mo^{VI}) DMSO reductase.[8] Only the pyranodithiolene parts of the two molybdopterin moieties (referred to as the P and Q molybdopterins) that coordinate molybdenum are shown for clarity.

impossibly acute bond angles. This later EXAFS study concluded that the crystallographic analyses were in error and that the correct active site was as suggested by the original EXAFS study.[7] This suggestion remained controversial until a later high-resolution crystallographic study showed cocrystallization of two different active site structures: one resembling the structure suggested by EXAFS and the other resembling one of the earlier crystal structures, which was attributed to an inactive enzyme.[8] The use of a HEPES buffer in the crystallization media was implicated in the conversion from the native to the (putatively) inactive form.[8] The active site structure of the oxidized enzyme is now generally agreed to be that shown in Figure 1B,[7,8] with coordination of the metal by Ser^{147}. Tyr^{114} has also been shown to be important in that it profoundly influences substrate specificity.[10] One remarkable finding in the first round of crystallographic studies was that of Bailey and coworkers, who found that when dimethyl sulfide (the product of catalytic turnover) was added to the oxidized enzyme, a form of the enzyme was produced in which DMSO was covalently bound to the molybdenum.[11] This form of the enzyme is of considerable interest because it is thought to be analogous to an intermediate of catalytic turnover. Both resonance Raman spectroscopy and X-ray absorption spectroscopy (XAS) indicate that the Mo atom is a des-oxo Mo^{IV} species,[6,7] but subsequent electronic spectra of both the dimethyl sulfide bound enzyme and the analogous species formed using dimethyl selenide were interpreted as supporting a $Mo^{V}\cdots OS^{\bullet}$ pair.[12] Our earlier EXAFS of this species[7] failed to detect any long-range interactions from the distant DMSO sulfur in the $Mo{-}O{=}S(CH_3)_2$ coordination assigned by crystallography presumably because the amplitude of this is below our detection limit.[7] We report herein further XAS studies on the dimethyl sulfide bound DMSO reductase and on the analogous complexes formed with dimethyl selenide and trimethylarsine. In addition, we show strong evidence that the species is best described as a Mo^{IV} complex and that the original crystallographic determination of the coordination of DMSO in this species is likely to be qualitatively correct, but with more conventional bond lengths for the bound DMSO.

Materials and Methods

Samples. Recombinant *R. sphaeroides* DMSO reductase was prepared and redox-cycled before use (to ensure that the active site was in the active form) as previously described.[7] Dimethyl selenide and trimethylarsine were obtained from Strem Chemicals Inc., and crystalline trimethylarsine oxide was a gift from Dr. Juergen Gailer, University of Calgary, Calgary, Canada. All other reagents were obtained from Sigma-Aldrich and were of the highest quality available. Sample preparations were done under an atmosphere of nitrogen in a 50 mM BICINE/NaOH buffer (pH 8.2). Solutions of trimethylamine, trimethylphosphine, dimethyl sulfide, dimethyl selenide, and trimethylarsine were prepared by sonicating the appropriate volume of the neat organic liquid in a buffer (50 mM BICINE/NaOH, pH 8.2). Dimethyl selenide and dimethyl sulfide have adequate solubilities, but trimethylarsine is only slightly soluble in water, and in this case, sonication formed a milklike emulsion of the compound, which on dilution dissolved immediately. In the case of trimethylarsine, addition of the reductant solution to the enzyme caused a color change from the characteristic greenish-gray-brown of the oxidized enzyme to a vivid pink. Dimethyl selenide produced an off-pink color, while trimethylphosphine produced a yellow-green solution and trimethylamine produced no change either in color or in XAS. Samples were transferred to 2 × 10 × 10 mm lucite sample cuvettes, rapidly frozen in cold isopentane at −140 °C, and then transferred to liquid nitrogen prior to XAS data collection.

XAS Data Collection. XAS measurements were conducted at the Stanford Synchrotron Radiation Laboratory with the SPEAR storage ring containing between 80 and 100 mA at 3.0 GeV. Mo K-edge data were collected on the structural molecular biology XAS beamlines 7-3 and 9-3, using wiggler fields of 1.8 and 2 T, respectively. Si(220) double-crystal monochromators were used, and in the case of beamline 7-3, harmonic rejection was achieved by offsetting the angle of the second monochromator crystal to 50% intensity at the end of the scan using an automated computer algorithm. Beamline 9-3 is equipped with a rhodium-coated vertical collimating mirror upstream of the monochromator and a downstream bent-cylindrical focusing mirror (also rhodium-coated). Harmonic rejection was accomplished by setting the cutoff angle of the mirrors to 23 keV. Incident and transmitted X-ray intensities were monitored using argon- or nitrogen-filled ionization chambers for molybdenum and arsenic data, respectively. X-ray absorption was measured as the Kα fluorescence excitation spectrum using

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(9) For the crystal site structure that showed an active site similar to that indicated by EXAFS analysis, the Mo−S ligation was in quantitative agreement with the EXAFS, whereas the Mo−O and Mo═O ligation differed slightly. We note that even in this high-resolution 1.3 Å structure the Mo═O ligand has overlapping van der Waals radii with a supposedly nonbonded atom (S_{13}), plus an impossibly acute bond angle O═Mo−S_{13} of 79°.[8] It seems possible that these remaining anomalies may be due to cocrystallization of multiple forms, perhaps with slightly different oxygen coordination. The fact that subtle structural variants of the active site structure can indeed occur is indicated by the two subtly different Mo^{V} EPR signals (called types 1 and 2) attributed to the reduced form of the structure in question, which we have previously characterized.[7]
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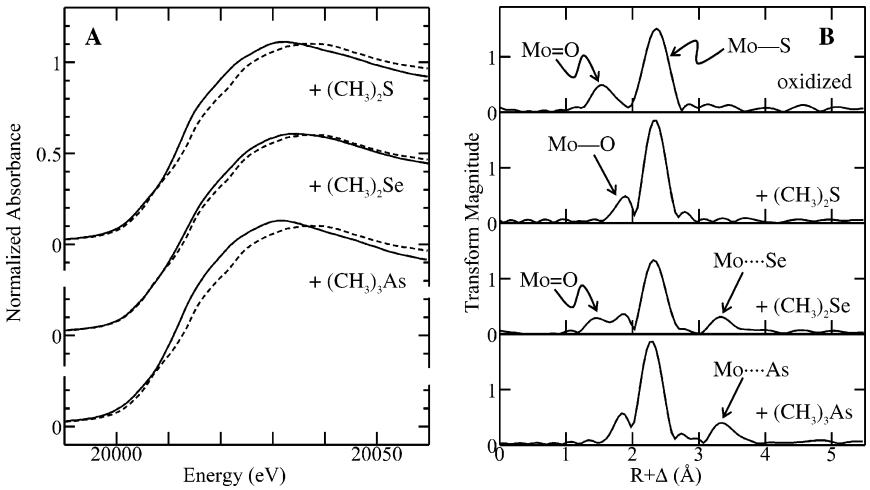

Figure 2. (A) Mo K-edge X-ray absorption near-edge spectra of DMSO reductase in the presence of 10 mM dimethyl sulfide, 10 mM dimethyl selenide, and 0.8 mM trimethylarsine (solid lines), compared with the spectrum of the oxidized enzyme (broken line). (B) EXAFS Fourier transforms (Mo−S phase-corrected) of the oxidized DMSO reductase and enzyme in the presence of dimethyl sulfide, dimethyl selenide, and trimethylarsine (concentrations are the same as those for A).

an array of 30 germanium detectors.[13] During data collection, samples were maintained at a temperature of approximately 10 K using an Oxford Instruments liquid-helium flow cryostat. For each sample, 8−12 scans were accumulated, and the energy was calibrated by reference to the absorption of a metal foil measured simultaneously with each scan, assuming lowest-energy inflection points of 20 003.9 and 11 867.0 eV for molybdenum and arsenic, respectively. The energy thresholds of the EXAFS oscillations were assumed to be 20 025.0 and 11 885 eV for molybdenum and arsenic, respectively.

XAS Data Analysis. The EXAFS oscillations $\chi(k)$ were quantitatively analyzed by curve-fitting over a k range of 1−14 $Å^{-1}$ using the *EXAFSPAK* suite of computer programs,[14] as described by George et al.,[15] using ab initio theoretical phase and amplitude functions calculated using the program *FEFF* version 8.25.[16,17] No smoothing, filtering, or related operations were performed on the data.

Molecular Modeling. Density functional theory (DFT) molecular modeling used the program *Dmol*^{3} *Materials Studio* version 3.2.[18,19] We expect bond-length accuracies of better than 0.05 Å and good estimates of energetic trends between postulated molecular entities. The Becke exchange[20] and Perdew correlation[21] functionals were used to calculate both the potential during the self-consistent-field procedure and the energy. Double-numerical basis sets included polarization functions for all atoms. Spin was unrestricted (automatically calculated), and all electron core potentials were used. No symmetry constraints were applied, and optimized geometries used energy tolerances of 2.0×10^{-5} hartree.

Results and Discussion

Near-Edge Spectra and EXAFS Fourier Transforms. Figure 2A compares the Mo K-near-edge spectrum of oxidized *R. sphaeroides* DMSO reductase with spectra obtained in the presence of dimethyl sulfide, dimethyl selenide, and trimethlyarsine. As expected from previous work and the absence of a color change, the trimethylamine spectrum is identical with that of the oxidized enzyme (not illustrated). Trimethylphosphine appears to be a special case and will be considered later. All three near-edge spectra of product-bound forms are shifted to lower energy relative to the oxidized enzyme (Figure 2). Near-edge spectra are comprised of transitions of the electron core (i.e., Mo 1s) to bound states involving the frontier molecular orbitals of the system and are sensitive to the electronic structure. The observed shift to lower energies (relative to the oxidized enzyme) of the near-edge spectra of the product-bound complexes suggests a lower oxidation state of molybdenum. We note that these shifts are small relative to the significant lifetime broadening of features at the Mo K-edge and that unambiguous determination of the oxidation states by examination of Mo K-near-edge spectra can often be problematic. The EXAFS Fourier transforms of these species are compared in Figure 2B. For the oxidized enzyme and the dimethyl sulfide treated enzyme, these data are equivalent to those reported earlier.[7] The Mo K-edge EXAFS is dominated by intense Mo−S backscattering from the four S atoms of the two dithiolene ligands,[5,7] which gives rise to the large Fourier transform peaks at about 2.3 Å observed in all four data sets (Figure 2B). For the oxidized enzyme, the transform peak at about 1.6 Å indicates the presence of Mo═O ligation, and its absence in the Fourier transform of the dimethyl sulfide bound EXAFS data indicates that this species has no terminal oxo ligand. The small peak at ∼1.9 Å is attributable to Mo−O ligation.[7] Bray and co-workers[12] have reported that treatment of oxidized *R. capsulatus* DMSO reductase with dimethyl selenide under conditions similar to those used here yielded a mixture of the oxidized enzyme plus a species similar to the dimethyl sulfide complex. The Fourier transform of dimethyl selenide treated DMSO reductase shown in Figure 2B is fully consistent with such a mixture (corresponding to approximately 50% oxidized

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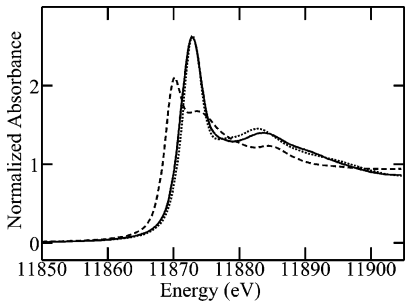

Figure 3. As K-edge X-ray absorption near-edge spectra of 0.8 mM DMSO reductase in the presence of stoichiometric trimethylarsine (solid line). The spectra of 1 mM solutions of trimethylarsine (dashed line) and trimethylarsine oxide (dotted line) are shown for comparison.

enzyme), as is the smaller energy shift observed for dimethyl selenide in the Mo K-near-edge spectrum (Figure 2A). Interestingly, the Fourier transform shows an outer-shell peak at ~3.4 Å, which we can attribute to a fractional Mo···Se interaction. With trimethylarsine, a similar ~3.4 Å transform peak is observed (assigned as a Mo···As interaction), while the remainder of the transform appears similar to that of the dimethyl sulfide species.

As we have noted, the Mo K-near-edge spectra are broad because of the short core−hole lifetime at the relatively high energies of the Mo K-edge; moreover, we cannot unambiguously discern the oxidation state of the Mo site from these data. Whereas dimethyl sulfide and dimethyl selenide both require an excess to form their respective enzyme complexes,[11,12] the spectrum given by trimethylarsine is identical whether stoichometric or excess trimethylarsine is used (not illustrated). This allows us to investigate the complex using XAS at the As K-edge as well as at the Mo K-edge. Figure 3 compares the As K-near-edge spectrum of trimethylarsine treated DMSO reductase with spectra of trimethylarsine and trimethylarsine oxide. The spectrum closely resembles that of trimethlyarsine oxide, which unambiguously indicates that the arsenic in the enzyme-bound species is in the fully oxidized As^V state. Thus, the trimethylarsine complex is best described as a Mo^{IV} species, which, in turn, suggests a formally reduced Mo^{IV} site for the dimethyl sulfide species. This result is in agreement both with the original assignment of Bailey and co-workers[11] and with our earlier work[7] and suggests that later suggestions[12] of Mo^V or even Mo^{VI} species are incorrect. More quantitative structural information on the nature of the active site structure is available from detailed curve-fitting analysis of the EXAFS portion of the spectrum.

EXAFS Spectroscopy and Curve-Fitting Analysis. Figure 4 shows both the EXAFS spectra and best fits at the Mo and As K-edges of the trimethylarsine complex, together with the corresponding EXAFS Fourier transforms. The parameters obtained from the curve-fitting analysis are summarized in Table 1. The Mo K-edge EXAFS curve-fitting analysis of the trimethlyarsine complex indicates four S ligands at 2.37 Å, which can be assigned to the two cofactor dithiolene ligands, two different Mo−O ligands at 2.01 and 2.23 Å, plus one Mo···As at 3.43 Å. These results are very similar to those obtained by EXAFS curve-fitting of the dimethyl sulfide complex, which gives four Mo−S at 2.37 Å and two different Mo−O interactions at 1.97 and 2.23 Å. We note that there is some uncertainty in the quantification of the longer of the two Mo−O moieties because of a rather high correlation with the Mo−S interaction in the refinement across part of the *k* range of the data. In agreement with our earlier analyses,[7] inclusion of a long-range Mo···S interaction did not significantly improve the fit, and it is clear that this interaction is not observed.[22,23]

The As K-edge EXAFS data clearly show both the As═O and As−C interactions that are expected for bound $(CH_3)_3$-As═O, and these are observed as transform peaks at approximately 1.7 and 1.9 Å, respectively (Figure 4). These data are in agreement with the near-edge spectra shown in Figure 3, which indicate an As^V species very similar to trimethylarsine oxide. The As K-edge EXAFS curve-fitting analysis indicates three As−C at 1.91 Å, one As═O at 1.70 Å, and an As····Mo interaction at 3.45 Å (Table 1), which is essentially the same as the Mo···As distance obtained from the Mo K-edge EXAFS. The structural parameters derived from analyzing the As K-edge EXAFS of a solution of trimethylarsine oxide (not illustrated) give As−C and As═O bond lengths of 1.90 and 1.67 Å, respectively. These are similar to those reported crystallographically for a calcium complex of trimethylarsine oxide,[24,25] which shows As−C and As═O bond lengths of 1.90 and 1.66 Å, respectively. A comparison of these molecules to the trimethylarsine DMSO reductase complex indicates essentially identical As−C bond lengths and a slightly longer As═O bond length of 1.70 Å, which is consistent with binding to molybdenum because subtle As═O elongation is expected from coordination of the metal. For example, Liu et al. have characterized a molybdenum complex of triphenylarsine oxide[26] that shows As−C and As═O bond lengths of 1.91 and 1.69 Å, respectively.

As discussed above, the dimethyl selenide complex does not form completely under the conditions employed by us or by Bray and co-workers, and a mixture with the oxidized enzyme is obtained.[12] Nevertheless, tentative conclusions on the active site structure can be formed by analysis of difference spectra. Figure 5 shows the result of subtracting 50% of the normalized spectrum of oxidized DMSO reductase from the normalized spectrum of a dimethyl selenide treated enzyme. The resulting XAS spectrum was then processed in the normal way to arrive at the data shown in

(22) We note that while others have previously claimed to observe such a distant sulfur [Baugh, P. E.; Garner, C. D.; Charnock, J. M.; Collison, D.; Davies, E. S.; McAlpine, A. S.; Bailey, S.; Lane, I.; Hanson, G. R.; McEwan, A. G. *J. Biol. Inorg. Chem.* **1997**, *2*, 634−643], the analysis methods employed by these workers were inadequate, and this conclusion should be discounted.[7]

(23) A possible contributing reason why the distance Mo···S is not observed may be EXAFS cancellation with the molybdopterin dithiolene C atoms. These are expected to lie at about the same distance from Mo as the S from bound DMSO (i.e., both at about 3.4 Å). Because Mo···S and Mo···C EXAFS are essentially 180° out of phase across the *k* range of our data, any Mo···C EXAFS might cause the amplitude of Mo···S to be reduced below the point where it can be detected. This is illustrated in the Supporting Information (Figure S2).

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(25) No crystal structure has been reported for pure trimethylarsine.

(26) Liu, C. M.; Nordlander, E.; Schmeh, D.; Shoemaker, R.; Pierpont, C. G. *Inorg. Chem.* **2004**, *43*, 2114−2124.

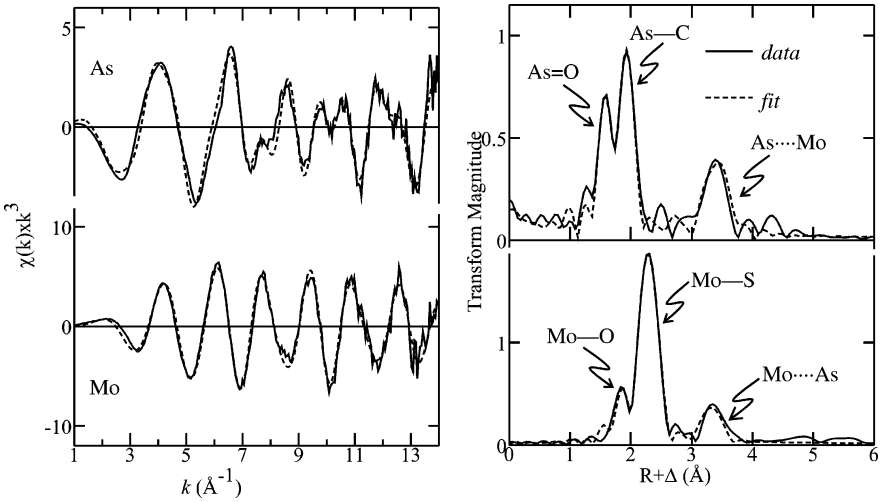

Figure 4. EXAFS at the Mo and As K-edges of DMSO reductase in the presence of stoichiometric trimethylarsine plus corresponding Fourier transforms (Mo−S and As−C phase-corrected, respectively). Solid lines show the experimental data and broken lines the results of EXAFS curve-fitting analysis (Table 1).

Table 1. EXAFS Curve-Fitting Parameters[a]

	Mo−S			Mo−O			Mo···As/Se				
sample	*N*	*R*	σ^2	*N*	*R*	σ^2	*N*	*R*	σ^2	ΔE_0	*F*
Me_3As	4	2.367(3)	0.0049(4)	1	2.01(1)	0.0035(7)	1	3.427(5)	0.0049(3)	−14.5(9)	0.215
				1	2.23(2)	0.0030(9)					
Me_2S	4	2.368(3)	0.0051(1)	1	1.98(1)	0.0025(2)				−14.8(5)	0.208
				1	2.229(9)	0.0037(2)					
Me_2Se[b]	4	2.378(3)	0.0059(4)	1	2.01(1)	0.0020(5)	1	3.404(3)	0.0037(3)	−14.6(9)	0.293
				1	2.28(2)[c]	0.0041(2)					
Me_3P	4	2.388(2)	0.0055(1)	1	1.730(2)	0.0025(2)				−16.4(4)	0.194

	As=O			As−C			As···Mo				
sample	*N*	*R*	σ^2	*N*	*R*	σ^2	*N*	*R*	σ^2	ΔE_0	*F*
Me_3As	1	1.699(2)	0.0021(2)	3	1.912(2)	0.0029(1)	1	3.450(3)	0.0056(2)	−7.6(4)	0.277

[a] Coordination numbers, *N*, interatomic distances *R* (Å), Debye−Waller factors σ^2 (Å^2), and threshold energy shifts ΔE_0 (eV). Values are quoted to the number of significant digits indicated by the estimated standard deviations (precisions) obtained from the diagonal elements of the covariance matrix, which are given in parentheses. The accuracies will be much greater than these values and are generally accepted to be ±0.02 Å for bond lengths and ±20% for coordination numbers and Debye−Waller factors. The fit-error function *F* is defined as $F = [\sum k^6(\chi_{calc} - \chi_{expt})^2/\sum \chi^2_{expt}]^{1/2}$, where the summations are over all data points included in the refinement. [b] Curve fitting of difference spectrum shown in Figure 5. We note that the structural parameters obtained by curve-fitting analysis will be affected by the exact difference spectrum used so the values reported here should be regarded as approximate. [c] We note that the normal limitation on interatomic distance resolution between similar scatterers is approximately given by $\pi/2\delta k$, where δk is the *k* range of the data. This does not apply between scatterers with significantly different phase functions, such as Mo−O and Mo−S.

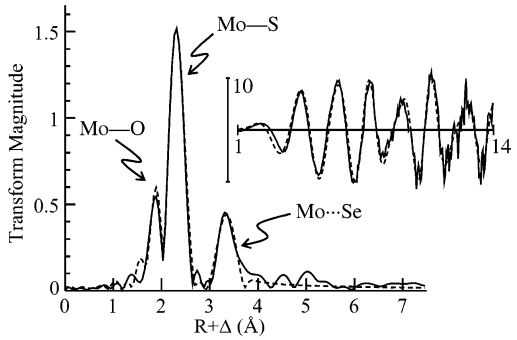

Figure 5. Mo K-edge EXAFS difference spectra (inset) and Fourier transform (Mo−S phase-corrected) generated by subtracting 50% of the normalized spectrum of oxidized DMSO reductase from the normalized spectrum of DMSO reductase treated with 10 mM dimethyl selenide (Figure 2B). Solid lines show the experimental data and broken lines the results of EXAFS curve-fitting analysis (Table 1).

Figure 5, which was subjected to curve-fitting analysis (Figure 5 and Table 1). We note that the exact quantity of the oxidized spectrum used to generate the difference spectrum will significantly affect the curve-fitting analysis, and any structural conclusions based on fitting difference spectra (Figure 5 and Table 1) should be regarded as approximate. Nevertheless, the similarities between the dimethyl selenide difference spectrum and the data from the trimethylarsine-reduced enzyme are obvious, and the results of curve fitting reinforce this conclusion.[27]

Figure 6 shows the DFT-energy-minimized model structure for the trimethylarsine-reduced DMSO reductase active site. The structure shows reasonable correspondence to the EXAFS-derived bond lengths, with DFT yielding slightly longer bond lengths (DFT typically overestimates bond lengths by up to 0.05 Å). Selected metrical parameters (DFT vs EXAFS) are compared in Table 2. The DFT structure does yield a significantly longer Mo−O($AsMe_3$) bond length (Table 2), which may indicate that the substrate binding pocket of the protein assists in stabilization of the trimethy-

(27) Because dimethyl selenide was added in significant excess, the corresponding Se K-edge data are unavailable.

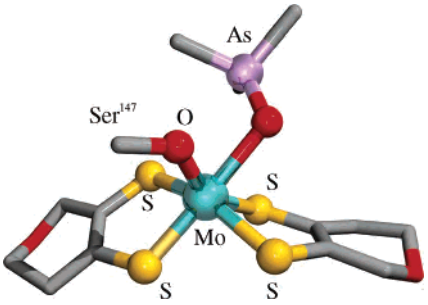

Figure 6. DFT-energy-minimized structure for the active site of trimethylarsine-reduced DMSO reductase. H atoms have been omitted for clarity.

Table 2. Comparison of Selected DFT- and EXAFS-Derived Parameters[a]

parameter	DFT	EXAFS
Mo−S	2.41	2.37
Mo−O (Ser^{147})	1.95	2.01
Mo−O ($AsMe_3$)	2.45	2.23
Mo···As	3.56	3.44[b]
As−O	1.68	1.70
As−C	1.96[c]	1.91
Mo−O−As	114	123[d]

[a] Bond lengths are given in angstroms and angles in degrees. [b] Average of As K-edge and Mo K-edge values. [c] Average value. [d] Computed from interatomic distances.

larsine oxide species. The longer value given by DFT for the Mo−O($AsMe_3$) bond and the correspondence of the Mo−O(Ser^{147}) bond length (Table 2) suggest that the longer of the two Mo−O bonds determined by EXAFS (Table 1) corresponds to bound trimethylarsine oxide.

Our results on the trimethylarsine-reduced enzyme have relevance to the conclusions derived from protein crystallography concerning the dimethyl sulfide bound *R. capsulatus* DMSO reductase.[11] DMSO is a common ligand in low-molecular-weight transition-metal species and can coordinate to the metal either via the S or via the O. A search of the Cambridge Structure Data Base[28] for free and oxygen-coordinated DMSO indicates that free DMSO has a S═O bond length of 1.50 Å, while DMSO bound to molybdenum via oxygen typically has a slightly longer S═O bond length (ca. 1.53 Å). The Mo−O(SMe_2) bond length in these complexes is invariably quite long, on average 2.29 Å; there is only minimal inverse correlation of this bond length with the S═O bond length (although a reasonable correlation of the Mo−O bond length with the Mo−O═S bond angle). This suggests that the longer of the two Mo−O bond lengths observed in the EXAFS is probably that from the coordinated substrate. The S═O bond length is thus only slightly elongated relative to that of free DMSO in the low-molecular-weight DMSO molybdenum species that have been characterized. Bailey and co-workers[11] observed that analysis of their crystallographic data using a DMSO molecule with a S−O interatomic distance that was restrained to normal bond lengths gave discrepancies with the electron density maps and that refinement with no restraints on the DMSO portion of the structure yielded an S−O bond length that was considerably elongated at 1.7 Å.[7] Such weakening of the S═O double bond through binding to molybdenum would be of significant interest in understanding the mechanism of catalytic turnover and for an isolated DMSO molecule would correspond to an energetic difference of some 0.5 eV.[29] It is increasingly realized that determining bond lengths directly is challenging for protein crystallography and thus subject to a degree of uncertainty.[30−32] Consequently, other techniques (such as XAS) can prove invaluable in providing complementary details that are lacking from protein crystallography. Any extrapolation of the results derived from the trimethylarsine-reduced enzyme to the more physiologically relevant dimethyl sulfide reduced enzyme critically depends upon the similarity of these two species. Studies of DMSO reductase have benefitted from the fact that this enzyme has molybdenum as the sole chromophore, whereas other molybdenum enzymes have intense electronic absorptions from other chromophores such as iron−sulfur clusters, heme, or flavin. Thus, electronic spectra can serendipitously be used to examine the active site in DMSO reductase. Figure 7A shows the electronic spectra of *R. sphaeroides* DMSO reductase in oxidized and various reduced forms. The spectrum observed for the dimethyl sulfide reduced enzyme is very similar to that reported previously by others,[11,12,33,34] having a characteristic absorption with a double peak at 485 and 546 nm. The trimethylarsine-reduced enzyme is broadly similar to that of the dimethyl sulfide reduced species, with the primary absorption in the visible having both a similar centroid and a similar extinction coefficient but with a single peak at 510 nm (with a small shoulder at approximately 571 nm) rather than the double peak observed with dimethyl sulfide. In both, the broad ~710 nm absorption characteristic of the oxidized enzyme is absent and the spectra are also quite distinct from that of the dithionite-reduced enzyme (Figure 7A). The electronic spectra thus reinforce our conclusion from the similarity of the EXAFS (see Figure 7B) that dimethyl sulfide reduced and trimethylarsine-reduced species are structurally similar. Thus, because we observe no unusually large distortion in the As−O bond length for trimethylarsine-reduced DMSO reductase, this strongly suggests that no unusual distortions are present in the S−O bond of dimethyl sulfide reduced DMSO reductase. This result disagrees with conclusions derived from resonance Raman spectroscopy, where Garton et al. assigned a band at 862 cm^{-1} as the S−O stretch of dimethyl sulfide reduced DMSO reductase (based on frequency and ^{18}O isotope shifts),[6] which is significantly shifted from the S−O stretch of free DMSO at 1003 cm^{-1}.[6] Despite the fact that a large number of low-molecular-weight DMSO compounds have been reported in the literature, there is very little data

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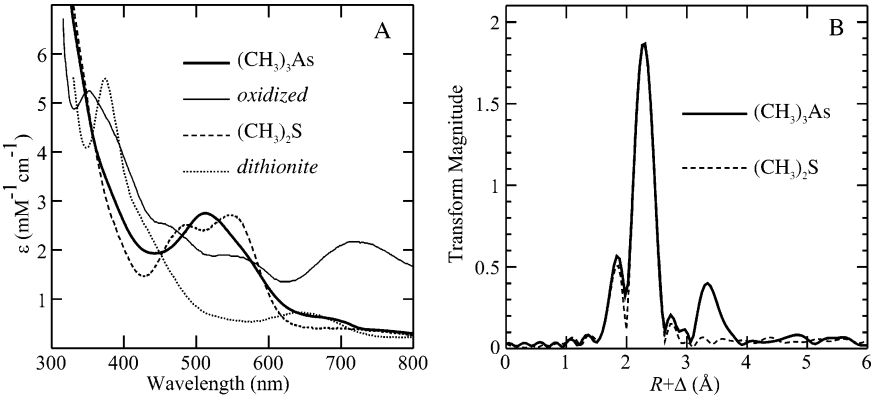

Figure 7. (A) UV−visible electronic spectra of DMSO reductase treated with 5 mM trimethylarsine, compared with spectra of the oxidized enzyme, of the enzyme treated with dimethyl sulfide, and of the dithionite-reduced enzyme. (B) Comparison of the EXAFS Fourier transforms (Mo−S phase-corrected) of trimethylarsine-reduced and dimethyl sulfide reduced DMSO reductase showing near-identity of the EXAFS for atoms directly coordinated to molybdenum.

on vibrational spectra. However, notwithstanding the absence of model data, it is hard to reconcile the assignment of a significantly perturbed S−O bond with our conclusions. Our interpretation of the XAS and electronic spectra suggests that either the dimethyl sulfide reduced and trimethylarsine-reduced enzymes have very different active sites (which we believe to be unlikely) or the 862 cm^{-1} resonance Raman band was incorrectly assigned. Bray et al.[12] have reported a Mo^{V} electron paramagnetic resonance (EPR) signal trapped by quickly freezing a reoxidation reaction of dimethyl sulfide reduced *R. capsulatus* DMSO reductase. This signal exhibits no detectible proton hyperfine splitting and is attributed to a species that has released DMSO, although the lack of proton hyperfine splitting might alternatively suggest that DMSO remains bound. We have observed essentially identical EPR signals with dimethyl sulfide reduced *R. sphaeroides* DMSO reductase (using the same reoxidation procedure as that of Bray et al.[12]). However, we could not obtain the analogous signal with trimethlyarsine because of the more rapid reoxidation for the arsenic complex, although the susceptibility to oxidation provides further support for the similarity of trimethylarsine-reduced and dimethyl sulfide reduced active sites.

Webster and Hall[35] have used DFT calculations to suggest partial bond formation between the S atom of DMSO and the O atom of Ser^{147} with a S···O bond of about 2.45 Å. With the trimethylarsine-reduced species, the As atom is essentially surrounded by methyl groups, so that in this case, a similar bond cannot form and thus such interactions cannot be important in stabilizing the trimethylarsine complex. If such S···O bonding does occur, it may be unimportant in the structurally characterized dimethyl sulfide reduced enzyme. We find that DFT minimizations using pyranodithiolene coordinates constrained at the crystallographic torsion angles invariably minimize to a structure that has dissociated DMSO, suggesting that the protein environment is important for stabilizing the structure of this intermediate. This observation is in agreement with the longer DFT value obtained for the Mo−O($AsMe_3$) bond length (see the discussion above). The possibility that dimethyl sulfide is not covalently bound to molybdenum must also be considered. As discussed above, the main evidence for dimethyl sulfide coordination is from a crystallographic study[11] that was very likely conducted on a heterogeneous sample containing fractional occupancies of active and inactive active sites.[8] Our EXAFS analysis failed to detect the sulfur of dimethyl sulfide; furthermore, our DFT calculations suggest that DMSO dissociation can readily occur. Thus, our study provides no direct evidence for coordination of dimethyl sulfide to the Mo site. It is possible that the conclusions of the crystal structure were confused by fractional occupancies in the active site or that the crystals contained coordinated dimethyl sulfide but in a species chemically distinct from the pink solution species that we and others have studied. However, we consider this latter possibility to be an unlikely one because of the vivid pink color reported for the crystals,[11] and dimethyl sulfide binding is supported by the similarities of the electronic spectra of the dimethyl sulfide species and the dimethyl selenide and trimethylarsine species, both of which have coordinated product.

As we have already noted, of the other five trimethyl group derivatives that were tested, trimethylamine did not interact with the wild-type enzyme while trimethylphosphine showed a quite distinct reaction, yielding a yellow-green rather than pink product. The near-edges (not illustrated) are quite similar to the dithionite-reduced enzyme but are distinct from those of the oxidized and dimethyl sulfide reduced enzymes. The observed similarity to the dithionite-reduced enzyme suggests that the trimethylphosphine-treated protein is also in the fully reduced Mo^{IV} oxidation state. The EXAFS Fourier transform plus the EXAFS and best fits are shown in Figure 8, and the results of the curve-fitting analysis are summarized in Table 1. Analysis clearly indicates the presence of a single Mo═O and four Mo−S ligands; however, inclusion of a Mo−O ligand, expected from ligation by Ser^{147}, did not improve the fit and resulted in large and physically unrealistic Debye−Waller factors,[7] effectively removing this contribution. Thus, reaction with trimethylphosphine appears to have caused dissociation of the amino acid ligand to molybdenum. We have previously suggested that the as-isolated recombinant enzyme (which is pale green) can have a related structure in that it apparently lacks Ser^{147} ligation but in the

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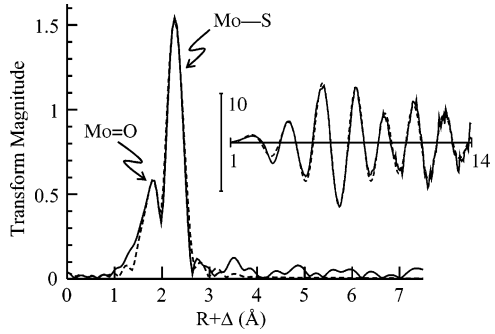

Figure 8. Mo K-edge EXAFS spectra (inset) and Fourier transform (Mo−S phase-corrected) of DMSO reductase treated with an excess (20 mM) of trimethylphosphine. Solid lines show the experimental data and broken lines the results of EXAFS curve-fitting analysis (Table 1).

Mo^{VI} oxidation state and possessing two Mo═O ligands.[7] This form lacks Mo^{V} EPR spectra, and the spectroscopically normal oxidized enzyme can be regenerated by a cycle of reduction and oxidation. A similar phenomenon has been observed with the $Cys^{207} \rightarrow Ser$ mutant of human sulfite oxidase, where Ser^{207} only ligates molybdenum after the enzyme is fully reduced.[36] We note in passing that our conclusions concerning the structure of as-isolated oxidized recombinant DMSO reductase have been criticized in studies using optical spectroscopy.[12,33,34] These workers have variously suggested that the as-isolated recombinant enzyme represents a mixture of inactive enzyme with a single dithiolene-bound and fully active enzyme or a species obtained from prolonged incubation of the dimethyl sulfide reduced enzyme in which DMSO is lost to form a des-oxo site.[12,33,34] We note that these assignments (especially the des-oxo DMSO-dissociated form) cannot be reconciled with our finding of a dioxo site by EXAFS or with our previous Mo^{V} EPR, which would have easily detected the presence of signals obtained from reducing an ordinary oxidized enzyme.

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In summary, we have presented a structural characterization of *R. sphaeroides* DMSO reductase reduced with trimethylarsine and show that this is structurally analogous to the physiologically relevant dimethyl sulfide reduced DMSO reductase. Our data unambiguously show that these species should be regarded as formal Mo^{IV} species and indicate a classical coordination complex of trimethylarsine, with no special distortions. Furthermore, we propose that the observed similarity of the trimethylarsine-reduced and dimethyl sulfide reduced enzymes is evidence that the dimethyl sulfide reduced enzyme possesses a classical coordination of DMSO with no unusual elongation of the S−O bond, as previously suggested.

Acknowledgment. Portions of this work were carried out at the Stanford Synchrotron Radiation Laboratory, which is funded by the U.S. Department of Energy, Office of Basic Energy Sciences and Office of Biological and Environmental Sciences, and the National Institutes of Health, National Center for Research Resources. Research at the University of Saskatchewan was supported, in part, by a Canada Research Chair award, the University of Saskatchewan, the Province of Saskatchewan, the National Institutes of Health (Grant GM5735), the Natural Sciences and Engineering Research Council of Canada (Grant 283315), and the Canadian Institute of Health Research. Research at Duke University is supported by the National Institutes of Health (Grant GM00091).

Supporting Information Available: Table of Cartesian coordinates for a DFT-minimized structure and figures showing EXAFS curve-fitting results for dimethyl sulfide reduced DMSO reductase and simulation of possible EXAFS cancellation for Mo···S(DMSO) and Mo···C(dithiolene) for dimethyl sulfide reduced DMSO reductase. This material is available free of charge via the Internet at http://pubs.acs.org.

IC0619052