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Structure of the Molybdenum Site of *Escherichia coli* Trimethylamine *N*-Oxide Reductase

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We report a structural characterization of the molybdenum site of recombinant *Escherichia coli* trimethylamine *N*-oxide (TMAO) reductase using X-ray absorption spectroscopy. The enzyme active site shows considerable similarity to that of dimethyl sulfoxide (DMSO) reductase, in that, like DMSO reductase, the TMAO reductase active site can exist in multiple forms. Examination of the published crystal structure of TMAO oxidase from *Shewanella massilia* indicates that the postulated Mo coordination structure is chemically impossible. The presence of multiple active site structures provides a potential explanation for the anomalous features reported from the crystal structure.

Introduction

The mononuclear molybdenum enzymes catalyze a range of reactions that in most cases involve two-electron redox chemistry coupled to the transfer of an oxygen atom to or from water. The enzymes all possess one or two molybdopterin dithiolene cofactors coordinated to the metal (Figure 1). 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. These are the xanthine oxidase family, the sulfite oxidase family, and the DMSO reductase family. Trimethylamine *N*-oxide (TMAO) reductase belongs to the DMSO reductase family of molybdenum enzymes, and BLAST searching results indicate that this enzyme is conserved in some of the α-proteobacteria and many of γ-proteobacteria, especially in the enterobacteria. The enzyme catalyzes the two-electron reduction of TMAO to trimethylamine (TMA)

$$\text{enzyme-Mo}^{IV} + (CH_3)_3N{=}O + 2H^+ \rightarrow \text{enzyme-Mo}^{VI} + (CH_3)_3N + H_2O$$

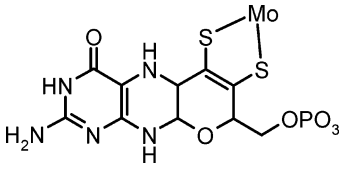

Figure 1. Schematic structure for the molybdopterin molybdenum cofactor.

TMAO is the major excretory nitrogen compound of fish and of some invertebrates.[2] The production of TMA by bacterial TMAO reductases is responsible for the characteristic fishy odor of decaying fish.

The crystal structure of TMAO reductase from *Shewanella massilia* has been reported at 2.5 Å resolution,[3] and an unusual seven-coordinate molybdenum site has been suggested, with two oxo groups, four sulfur ligands, and one longer Mo−O bond (Figure 2). This structure is very similar to that reported for oxidized *Rhodobacter capsulatus* DMSO reductase by Bailey and co-workers.[4] The active site structures of the *Rhodobacter sphaeroides*[5] and *R. capsulatus*[4,6] enzymes were previously the subject of a controversy. Crystallographic studies by three different groups suggested dramatically different active site structures, within essentially

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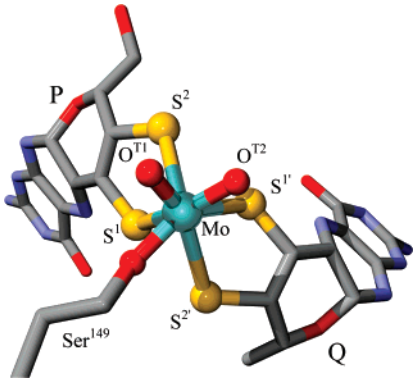

Figure 2. Crystal structure for the active site of oxidized (Mo^{VI}) *S. massilia* TMAO reductase[3] showing the proposed seven-coordinate active site.

identical polypeptide folds. All of the crystallographic conclusions were inconsistent with previous extended X-ray absorption fine structure (EXAFS) spectroscopy[7] and with subsequent resonance Raman spectroscopy.[8] Moreover, a later EXAFS study showed that the active site structures proposed from crystallography were all substantially chemically unreasonable.[9] For example, atoms that were supposedly nonbonded had overlapping van der Waals radii, and structures contained impossibly acute bond angles. This study concluded that the crystallographic analyses were in error and that the correct active site resembled that suggested by the original EXAFS study.[7] This suggestion remained controversial until a later high-resolution crystallographic study[10] 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 in which one of the two molybdopterins is dissociated from Mo.[10] This study directly confirmed the presence of multiple structures of the active site, which had previously been demonstrated spectroscopically,[11] and that one of the two molybdopterins might dissociate in some forms of the enzyme had already been suggested in the first EXAFS study.[7] Finally, recent in situ EXAFS experiments on the *Escherichia coli* DMSO reductase still contained within its native membranes (and thus more likely to be in a physiologically relevant form) indicated an active site structure similar to that proposed from the original EXAFS experiments.[12] While the controversy on DMSO reductase is now resolved, the active site structure reported for TMAO reductase remains an anomaly in the literature. We report herein a re-examination of the active site structure of TMAO reductase using X-ray absorption spectroscopy and show that like DMSO reductase, the active site can exist in different forms.

Materials and Methods

Samples. Recombinant *E. coli* TMAO reductase was prepared as previously described.[13] All other reagents were obtained from Sigma-Aldrich and were of the highest quality available. Sample preparations were performed under an atmosphere of nitrogen in 50 mM BICINE/NaOH buffer, pH 8.2. To prepare dithionite-reduced samples, 5 mM (final) dithionite with 10 μM (final) methyl viologen was incubated with the sample for 1 min. Samples were transferred to (2 mm × 10 mm × 10 mm) lucite cuvettes, which were 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. Molybdenum K-edge data were collected on the structural molecular biology XAS beamline 7-3. A Si(220) double-crystal monochromator was used, and harmonic rejection was achieved by offsetting the angle of the second monochromator crystal to 50% intensity at the end of the scan. Incident and transmitted X-ray intensities were monitored using argon-filled ionization chambers. The X-ray absorption was measured as the Kα fluorescence excitation spectrum using an array of 30 germanium detectors.[14] During data collection, samples were maintained at a temperature of approximately 10 K using an Oxford Instruments liquid helium flow cryostat. For each sample, eight to 12 scans were accumulated, and the energy was calibrated by reference to the absorption of a molybdenum foil measured simultaneously with each scan, assuming a lowest energy inflection point of 20 003.9 eV. The energy threshold of the extended X-ray absorption fine structure (EXAFS) oscillations was assumed to be 20 025.0 eV.

XAS Data Analysis. The EXAFS oscillations $\chi(k)$ were quantitatively analyzed by curve-fitting using the EXAFSPAK suite of computer programs[15] as described by George et al.,[16] using ab initio theoretical phase and amplitude functions calculated using the program FEFF version 8.25.[17,18] No smoothing, filtering, or related operations were performed on the data.

Molecular Modeling. Density functional theory (DFT) molecular modeling using the program Dmol[3] Materials Studio Version 4.1 was performed.[19,20] We expect bond length accuracies of better than 0.05 Å and good estimates of energetic trends between postulated molecular entities. The Becke exchange[21] and Perdew correlation[22] 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. Calculations were spin unrestricted, and all electron core potentials were used. No

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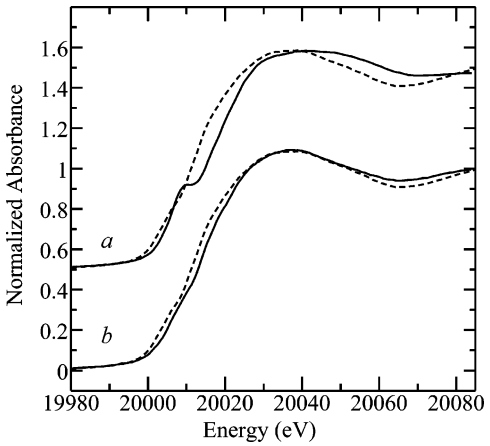

Figure 3. Molybdenum K-edge X-ray absorption near-edge spectra of *E. coli* TMAO reductase. (a) As-isolated enzyme (solid line) as compared to dithionite-reduced enzyme (dashed line) and (b) redox-cycled enzyme (solid line) as compared to dithionite-reduced enzyme (dashed line).

symmetry constraints were applied, and optimized geometries used energy tolerances of 2.0×10^{-5} Hartree.

Results and Discussion

Figure 3 shows the Mo K-edge near-edge spectra of different forms of TMAO reductase studied in this work. Near-edge spectra are comprised of transitions of the core−electron (Mo 1s in our case) to bound states involving the frontier molecular orbitals of the system and are sensitive to electronic structure. Figure 3a compares the spectrum of an oxidized as-isolated enzyme with the spectrum of a dithionite-reduced enzyme, and Figure 3b compares the spectrum of reduced enzyme that has been reoxidized by adding an excess (10 mM) of trimethylamine *N*-oxide, with the spectrum of dithionite-reduced enzyme. As expected, the spectrum of the predominantly Mo^{IV}-reduced enzyme is shifted to lower energy relative to the spectra of the oxidized Mo^{VI} samples. Significantly, the spectrum of as-isolated enzyme is quite distinct from that of reoxidized enzyme. In particular, the as-isolated data show a distinct peak[23] in the near-edge at around 20 009.7 eV. This pre-edge feature is attributed to a formal Mo 1s → Mo═O π^* transition[24] and is characteristic of the presence of Mo═O ligation,[25] and its intensity is related to the number of Mo═O ligands.[26−28] The strong presence of this feature in the as-isolated enzyme, together with a weak presence for the reduced and reoxidized enzyme, suggests a greater number of Mo═O ligands in the former sample. Reoxidation of reduced DMSO reductase (called redox cycling) has been shown to restore a damaged active site to its active structure,[9] and the near-edge data suggest that a similar process can occur with TMAO reductase. No spectral changes[29] were observed when low molecular weight species were removed from redox-cycled enzyme by gel-filtration (not illustrated). Also, unlike DMSO reductase,[30] no changes in the XAS or UV−vis spectra of oxidized enzyme were observed when excess product (trimethylamine) was added,[31] suggesting that product complexes do no readily form with TMAO reductase.

More quantitative structural information on the metal coordination is available from analysis of the EXAFS portion of the spectrum. Figure 4 compares the Mo K-edge EXAFS oscillations of the as-isolated enzyme, dithionite-reduced enzyme, and redox-cycled enzyme, together with the best fits and EXAFS Fourier transforms. The results of curve-fitting analysis are given in Table 1. The as-isolated enzyme clearly shows two Mo═O interactions at 1.72 Å, which give rise to the intense Fourier transform peak at ∼1.6 Å (Figure 4). The active site is completed by two Mo−S donors at 2.45 Å and a long Mo−O bond at 1.95 Å, which combine to give the transform peak at about 2.3 Å. No Mo═O trans effects are detectable in the Mo−S bond lengths.[9] Interestingly, the Mo coordination structures of the recombinant as-isolated forms of *R. sphaeroides* DMSO reductase,[9] *R. sphaeroides* biotin sulfoxide reductase,[32] and *E. coli* TMAO reductase (present work) have all been different from one another. The basis for this remains to be determined. The EXAFS of dithionite-reduced enzyme indicates a *des*-oxo Mo site with the Fourier transform lacking the characteristic Mo═O transform peak in (Figure 4). The EXAFS can be adequately fit with just four Mo−S ligands at 2.35 Å, but just four such bonds at that distance is not chemically reasonable, and a somewhat better fit is obtained with a combination of Mo−S and Mo−O interactions. In particular, four Mo−S bonds at 2.34 Å plus one Mo−O bond at 1.90 Å and one Mo−O bond at 2.23 Å give an excellent fit (Table 1). We note that these two Mo−O interactions produce EXAFS that is close to cancellation, which explains why four Mo−S bonds fit reasonably well. It also gives rise to a large error in the absolute values of these bond lengths. The EXAFS data of the redox-cycled enzyme indicate a single Mo═O ligand at 1.71 Å, four Mo−S ligands at 2.43 Å, and one Mo−O ligand at 1.83 Å (Table 1).

These analyses allow us to postulate structures for the different active site forms, and these are shown in Figure 5. These structures are remarkably similar to the different active sites postulated for DMSO reductase,[7] with the as-isolated form being analogous to the (supposedly) inactive form of DMSO reductase characterized by Schneider et al.,[6] in which

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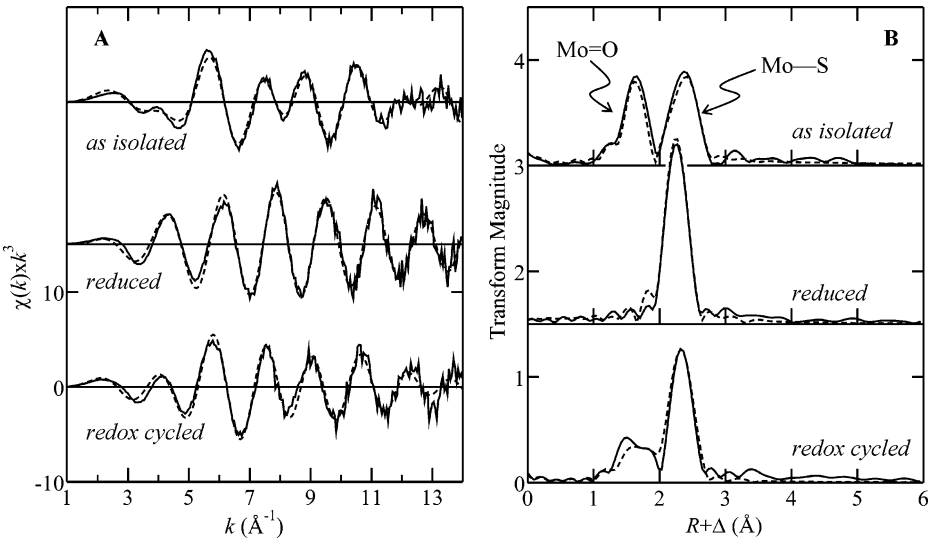

Figure 4. (A) EXAFS oscillations (solid lines) and best fits (dashed lines) of as-isolated, dithionite-reduced, and redox-cycled enzyme. (B) EXAFS Fourier transforms (Mo−S phase-shift corrected) corresponding to the data shown in panel A. The transform peaks that are indicative of Mo═O and Mo−S contributions are shown (note that any Mo−O transform peaks will be underneath the Mo−S peak).

Table 1. Trimethylamine *N*-Oxide Reductase EXAFS Curve-Fitting Results[a]

	Mo═O			Mo-S			Mo-O				
	N	*R*	σ^2	*N*	*R*	σ^2	*N*	*R*	σ^2	ΔE_0	*F*
as-isolated[b]	**2**	**1.716(3)**	**0.0038(2)**	**2**	**2.450(3)**	**0.0050(2)**	**1**	**1.948(8)**	**0.0057(8)**	**−13.8(4)**	**0.305**
	2	1.723(3)	0.0046(2)	2	2.470(3)	0.0040(2)				−14.0(5)	0.323
				2	2.454(5)	0.0049(5)				*−15.5(5)*	*0.797*
reduced				**4**	**2.336(1)**	**0.0047(1)**	**1**	**1.90(2)**	**0.0057(8)**[c]	**−14.3(6)**	**0.267**
							1	2.23(2)	**0.0057(8)**[c]		
				4	2.337(2)	0.0043(1)	1	1.892(5)	0.0060(8)[d]	−16.7(5)	0.293
redox-cycled[b]	**1**	**1.705(6)**	**0.0048(9)**	**4**	**2.431(3)**	**0.0065(2)**	**1**	**1.832(9)**	**0.0071(8)**[d]	**−14.8(3)**	**0.377**
	1	1.728(5)	0.0051(6)	4	2.431(2)	0.0063(2)				−14.9(4)	0.386

[a] Coordination numbers, *N*, interatomic distances *R* (Å), Debye−Waller factors σ^2 (Å^2), and threshold energy shift ΔE_0 (eV). Values in bold represent the best fits obtained for a particular species. Values in parentheses are the estimated standard deviations obtained from the diagonal elements of the covariance matrix. We note that these are precisions and are distinct from the accuracies that are expected to be larger (ca. ±0.02 Å for *R* and ±20% for *N* and σ^2). The amplitude scale factor, otherwise known as the many-body amplitude reduction factor, or S_0^2, was defined by fitting data from a number of model compound species as 1.05. The fit-error function *F* is defined as $\{\Sigma k^6(\chi_{calcd} - \chi_{exptl})^2/\Sigma\chi_{exptl}^2\}^{1/2}$, where the summations are over all data points included in the refinement. [b] When the Mo═O group was omitted from the fit, the Mo−O bond length converged to the short values characteristic of Mo═O ligation. [c] These Debye−Waller factors were found to be highly correlated in the fit, so they were held to a common value and constrained within chemically reasonable bounds.[9] [d] This parameter was constrained to within chemically reasonable bounds.[9]

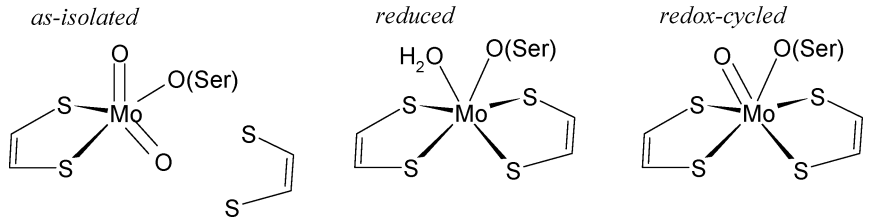

Figure 5. Postulated structures for as-isolated, dithionite-reduced, and redox-cycled enzyme.

one pterin dithiolene is dissociated from Mo, whereas the redox-cycled mono-oxo form is analogous to the functional Mo^{VI} form of DMSO reductase.[7,9,10] Examination of the as-isolated enzyme from different (independent) preparations of TMAO reductase indicates that the active site was generally a mixture of di-oxo and mono-oxo forms, with proportions varying from preparation to preparation (not illustrated). Redox cycling converted the active site into a form that gave XAS data essentially identical to that shown in Figures 3 and 4. Our data thus suggest that the active site of TMAO reductase can exist in the different forms shown in Figure 5, with a considerable resemblance to DMSO reductase.

These structures are significantly different from the active site coordination proposed by Czjzek et al.[3] While it is possible that structural differences may arise from differences between the *E. coli* and the *S. massalia* enzymes, this seems unlikely as BLAST results show that the enzymes share 49% sequence identity and 67% sequence similarity. In particular, 24 of the 35 amino acids that are suggested to interact with the molybdopterin cofactor in *S. massilia* are conserved in *E. coli*.[3] Thus, it seems very likely that the TMAO reductases from two organisms share a common structure for the molybdenum center. Sequence similarities alone cannot definitively prove that the active site structures of the *S. massilia* and *E. coli* TMAO reductases have similar active

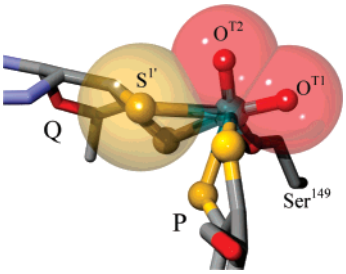

Figure 6. Crystal structure of oxidized (Mo^{VI}) *S. massilia* TMAO reductase[3] showing overlapping van der Waals radii (translucent spheres) for three supposedly nonbonded atoms (S1′, O^{T1}, and O^{T2}).

sites, and the possibility remains that the enzymes have significantly different active sites. However, scrutiny of the *S. massilia* TMAO reductase active site structure proposed from crystallography[3] reveals some chemically impossible features. First, the active site contains several anomalous bond lengths; the Mo−O(Ser149) bond length at 1.7 Å is too short for a Mo−O single-bond coordination,[33] and the four Mo−S bond lengths are all rather longer than expected, at 2.5, 2.6, 2.7, and 2.8 Å.[33] Such bond length discrepancies are not completely unexpected, as metalloprotein active sites are notoriously difficult to determine with chemically significant accuracy.[34] A more serious problem is that the proposed molybdenum coordination of the active site is much too crowded. Thus, the distances between several supposedly nonbonding atoms is significantly shorter than the sum of their van der Waals radii (Figure 6), and some bond angles are unreasonably small. For example, O^{T1}═Mo═O^{T2} is only 80°, and O^{T2}═Mo−S1′ is only 73°. Di-oxo Mo sites are a very common structural motif. Of 1185 *cis*-dioxo Mo^{VI} species in the Cambridge Crystal Structure Database, there are no valid structures with O═Mo═O bond angles of less than 90° and a minimum bona fide value of 95° for a *cis*-dioxo-tetraphenylporphyrinato Mo^{VI} species.[35] In this example, the normally rigidly planar porphyrin ring system is heavily distorted from the strain imposed by maintaining the *cis*-dioxo bond angle, with the pyrrole rings alternating in tilts that are up and down relative to the mean porphyrin plane.[35] In agreement with small molecule crystallography, DFT calculations yield an ideal bond angle of 104.4° (as compared to the mean of 105.2° from the Cambridge Crystal StructureDatabase) and furthermore indicate that the energetic penalty in decreasing this to 80° is quite large, at about 1.2 eV. Upon submitting the active site structure to DFT energy minimization, the energy decreases by a staggering 8.4 eV, sufficient to break most covalent bonds. It is thus clear that the metrics of the crystallographically derived active site are unreasonable. It seems likely that this apparent overcrowding of the TMAO reductase active site is due to the cocrystallization of multiple forms of the enzyme. In this case, the active site atoms will be a superposition of partially occupied positions. Such fractional occupancy might easily be missed in a protein structure, especially at poorer resolutions, and would give rise to increased temperature factors for the atoms concerned, and in agreement with this, the temperature factors for several active site atoms are high, and in particular, O^{T1} and O^{T2} have reported isotropic temperature factors of 20 and 18 Å,[2] respectively.[3]

In summary, the conclusions of the crystal structure of the TMAO reductase[3] do not structurally agree with our XAS results and furthermore are chemically unreasonable. The XAS indicate an active site that can exist in multiple forms and is substantially similar to that of DMSO reductase. The phenomenon of multiple forms cocrystallizing, interpreted as a single species, may explain the anomalous active site structure for TMAO reductase that has been proposed from protein crystallography.

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