The Molybdenum Site of Sulfite Oxidase: A Comparison of Wild-Type and the Cysteine 207 to Serine Mutant Using X-ray Absorption Spectroscopy

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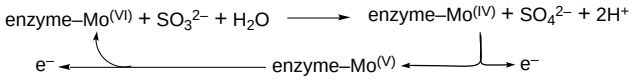
Abstract: X-ray absorption spectroscopy at the molybdenum and sulfur K-edges has been used to probe the active site of wild-type and cysteine 207 → serine mutant human sulfite oxidases. We compare the active site structures in the Mo(VI) oxidation states: the wild-type enzyme possesses two Mo═O ligands at 1.71 Å and three Mo−S ligands at 2.41 Å. The mutant molybdenum site is a novel trioxo site with Mo═O bond lengths of 1.74 Å, with two Mo−S ligands at 2.47 Å. We conclude that cysteine 207 is a ligand of molybdenum in wild-type human sulfite oxidase, and that, in the mutant, the Mo is ligated to an extra oxo group rather than the hydroxyl of the substituent serine 207.

Introduction

Sulfite oxidase is an oxo-transferase which is responsible for the physiologically vital oxidation of sulfite to sulfate.[1] Residing in the mitochondrial inter-membrane space, the enzyme is dimeric with a subunit mass of about 52 000. Each monomer contains molybdenum associated with a single pterin cofactor, and a cytochrome *b* type heme. The two-electron oxidation of sulfite to sulfate is known to occur at the molybdenum site, which is reduced from Mo(VI) to Mo(IV) in the process, and the catalytic cycle is completed with re-oxidation of the molybdenum first to Mo(V) and then to Mo(VI), by intramolecular electron transfer to the cytochrome *b* site.[2,3]



With the exception of nitrogenase, all molybdenum enzymes that have been described to date[4] contain a novel pterin cofactor in which the molybdenum is bound by the dithiolene side chain of the pterin ring[5] (see Figure 1). Until very recently,[6] structural information on molybdenum enzymes has derived almost entirely from spectroscopy of the enzymes and of model compound systems.[4] As one of the most intensively studied molybdenum enzymes,[4] sulfite oxidase can be regarded as the prototypical member of one class of molybdenum enzymes, those possessing dioxo molybdenum sites when the enzyme is in the fully oxidized Mo(VI) form.[7,8]

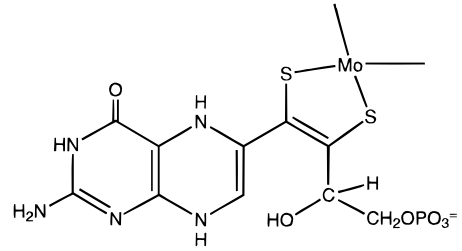


Figure 1. Proposed minimal structure of the molybdenum cofactor. The reversible formation of tricycle via the formation of a pyran ring through attack of the 3′-OH at C-7 of a dihydropterin has been proposed [*e.g.* see ref 6]. In enzymes from prokaryotic sources the cofactor has a 5′GMP moiety attached via a pyrophosphate linkage.

In earlier EXAFS studies,[8] the Mo−S coordination number of sulfite oxidase was judged to be three. While this conclusion was bordering on the limits of accuracy of the EXAFS technique, it suggested the existence of an extra thiolate ligand from the protein, in addition to the two expected from the dithiolene side chain of the pterin cofactor (Figure 1).[6] Sulfite oxidase contains four cysteine residues, of which one, Cys207, is conserved in rat, chicken, and human sulfite oxidase and in nitrate reductases from several fungal and plant sources.[9] Recently rat and human sulfite oxidase genes have been successfully cloned and expressed heterologously in *Escherichia*

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coli. Cysteine to serine mutants for all of the cysteine residues in the rat enzyme have been constructed.[10] Of these, only the cysteine 207 to serine (C207S) mutant had modified activity (reduced from 2300 units/mg in the wild-type to <2 units in the mutant).[10] Nevertheless, the C207S mutant proteins of both the rat and human enzyme contain a full complement of both pterin and molybdenum.[10] We report herein an XAS study of the molybdenum active site of both wild-type and C207S mutant human sulfite oxidases in the oxidized Mo(VI) oxidation state.

Materials and Methods

Samples. Recombinant human sulfite oxidase was purified as described by Garrett and Rajagopalan.[10] Samples for XAS were prepared at a final concentration of approximately 1.0 mM Mo in a mixed buffer system consisting of 20 mM Tris, bis-Tris, and bis-Tris−propane pH 9.0, with no added chloride. For molybdenum K-edge measurements, samples were frozen in 10 mm × 10 mm × 3 mm lucite sample curvettes. For sulfur K-edge measurements, samples were prepared as lyophylized powders, with other experimental conditions as described by George *et al*.[11]

XAS Data Collection. XAS measurements were carried out at the Stanford Synchrotron Radiation Laboratory with the SPEAR storage ring containing 55−90 mA at 3.0 GeV.

Molybdenum K-edge data were collected on beamline 7-3 using a Si(220) double crystal monochromator, with an upstream vertical aperture of 1 mm, and a wiggler field of 1.8 T. Harmonic rejection was accomplished by detuning one monochromator crystal to approximately 50% off peak, and no specular optics were present in the beamline. The incident X-ray intensity was monitored using an argon-filled ionization chamber and X-ray absorption was measured as the X-ray Mo Kα fluorescence excitation spectrum with an array of 13 germanium detectors through a zirconium metal filter of 3 absorption thickness, and a Soller slit assembly.[12] Samples were maintained at a temperature of approximately 10 K during data collection, using an Oxford Instruments liquid helium flow cryostat. Sixteen 35-min scans were accumulated for each sample, and the absorption of a molybdenum metal foil was measured simultaneously by transmittance. The X-ray energy was calibrated with reference to the lowest energy inflection point of the molybdenum foil spectrum, which was assumed to be 20003.9 eV.

Sulfur K-edge data were collected on beamline 6-2 using a Si(111) double-crystal monochromator, and a wiggler field of 1.0 T. Harmonic rejection was accomplished by a flat nickel-coated mirror downstream of the monochromator, adjusted to have a cutoff energy of about 4500 eV. Energy resolution was optimized by decreasing the vertical aperture upstream of the monochromator until no further sharpening of features of the near-edge spectrum of a sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) standard was detected (we estimate an approximate resolution of 0.5 eV). Residual undulator structure from the 54-pole insertion device was minimized by vertically translating the aperture to a position just off the peak of maximum intensity. The experiment was conducted at room temperature and three 20-min scans were averaged for both mutant and wild-type sulfite oxidases. Longer exposures to the X-ray beam caused photoreduction of the sample, as judged by changes in the Mo L-edge spectra (not illustrated). The X-ray fluorescence was monitored using a Stern-Heald-Lytle fluorescent ion chamber detector[13] and the energy scale was calibrated with reference to the lowest energy peak of the sodium thiosulfate standard which assumed to be 2469.2 eV.[14]

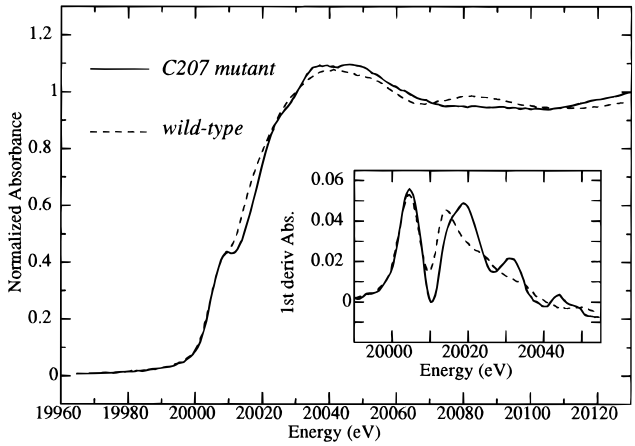

Figure 2. Molybdenum K-edge near-edge spectra of C207S mutant (solid line) and wild-type (broken line) sulfite oxidase. The inset shows a first derivative of the spectra.

Data Analysis. The extended X-ray absorption fine structure (EXAFS) oscillations $\chi(k)$ were quantitatively analyzed by curve-fitting with the EXAFSPAK suite of computer programs[15] using *ab initio* theoretical phase and amplitude functions generated with the program *feff* version 6.01.[16] The sulfur K-edge spectra were subjected to a rigorous normalization algorithm wherein the portions of the data above and below the near-edge region (*i.e*. excluding the most structured part) were fitted to the X-ray cross-sections tabulated by McMaster[17] by refining the coefficients of a polynomial background function and a scaling factor. No smoothing or related manipulation was performed upon the data.

Results and Discussion

Molybdenum K-edge Near-Edge Spectra. Figure 2 shows the Mo K-edge near-edge spectra of the wild-type and C207S mutant sulfite oxidases. The spectra are broadly similar, but with significant differences that are highlighted by the derivative plot shown in the inset. The spectrum of the mutant is shifted by approximately 1.5 eV to higher energy (see the derivative plot). The spectrum of the wild-type human enzyme is very similar to that previously reported for chicken liver sulfite oxidase.[8]

The Mo near-edge spectra of both mutant and wild-type proteins have pronounced pre-edge features at about 20008 eV. This is the so-called "oxo-edge" feature which is characteristic of a species possessing Mo═O groups, or to a lesser extent Mo═S;[18,19] it arises from formally dipole forbidden 1s → 4d bound-state transitions to antibonding orbitals directed principally along Mo═O bonds.[18] This bound-state transition is significantly more intense in the spectrum of the mutant[20] (see

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(20) Deconvolution of the spectra by curve-fitting to a sum of four pseudo-Voigt peaks plus an integrated pseudo-Voigt step function [using the EXAFSPAK program EDG-FIT *e.g*. see ref 21] gave peak areas of 0.71 and 1.61 for the 20008-eV transition of the wild-type and mutant, respectively.

Table 1. EXAFS Curve Fitting Results[a]

Mo−O			Mo−S			Mo−O					
N	*R*	σ^2	*N*	*R*	σ^2	*N*	*R*	σ^2	E_0	*V*	error[b]
Wild-Type											
1	1.715(3)	0.0003(3)	3	2.411(4)	0.0046(2)				−13.9(10)	4.1	0.409
2	1.709(3)	0.0030(2)	2	2.405(3)	0.0027(2)				−14.9(10)	5.0	0.345
2	**1.712(3)**	**0.0030(2)**	**3**	**2.411(3)**	**0.0047(2)**				**−13.8(8)**	**5.9**	**0.331**[c]
2	1.714(3)	0.0031(2)	4	2.415(4)	0.0065(2)				−13.0(8)	6.6	0.370
3	1.712(3)	0.0053(2)	3	2.409(3)	0.0047(2)				−13.9(8)	7.5	0.352
C207S											
1	1.743(1)	−0.0002(2)	2	2.471(6)	0.0042(3)				−13.6(13)	2.9	0.476
2	1.744(2)	0.0023(2)	2	2.474(4)	0.0042(2)				−12.7(8)	4.4	0.307
2	1.742(3)	0.0023(2)	2	2.472(4)	0.0042(2)	1	2.5(1)	0.028(20)	−13.4(9)	4.7	0.305[d]
2	1.745(3)	0.0029(2)[e]	2	2.475(3)	0.0041(2)	1	2.0[f]	0.0029(2)[e]	−12.9(6)	5.3	0.433
2	1.772(1)	0.0014(3)[g]	2	2.474(2)	0.0043(2)	1	1.671(4)	0.0014(3)[g]	−12.9(6)	6.1	0.257[h]
3	**1.744(2)**	**0.0043(1)**	**2**	**2.473(3)**	**0.0043(2)**				**−12.9(6)**	**6.0**	**0.262**[c]
4	1.743(3)	0.0062(2)	2	2.471(3)	0.0045(2)				−12.9(6)	7.5	0.313

[a] Coordination number *N*, interatomic distance *R* (Å), (thermal and static) mean-square deviation in *R* (the Debye−Waller factor) and σ^2 (Å^2), E_0 (eV) the threshold energy shift, and *V* the bond-valence sum calculated from *N* and *R*.[27,28] Values in parentheses are the estimated standard deviations (precisions) obtained from the diagonal elements of the covariance matrix. We note that the accuracies will always be somewhat larger than the precisions, typically ±0.02 Å for *R* and ±25% for *N* and σ^2. Coordination numbers *N* were not refined, but held fixed at the values shown, values for *R*, σ^2, and E_0 were refined. The *k* range for the fitting was from 1 to 14.5 Å^{-1} as shown in Figure 3. [b] The fit-error is defined as $\sum k^6(\chi_{exptl} - \chi_{calcd})^2/\sum k^6\chi_{exptl}^2$. [c] Fits shown in bold type represent the conclusion for the sample. [d] We note that the refinement procedure has effectively removed the Mo−O contribution (comprising only 0.2% of the total) by increasing the bond length to 2.5 Å (from an initial value of 2.0 Å), and the Debye−Waller to a physically unrealistic extent. [e] The values of these Debye−Waller factors were constrained to be equal. [f] The parameter was restrained at the value shown. [g] The values of these Debye−Waller factors were constrained to be equal as they were found to be very highly correlated in the fit. [h] This fit has a lower fit-error than any other for this sample, but the difference in bond length is very close to the EXAFS resolution as discussed in the text.

the inset to Figure 2). The presence of the "oxo-edge" feature in both spectra argues for the existence of Mo═O ligands in both of the sulfite oxidase samples.

Molybdenum K-edge EXAFS Spectra. Figure 3 shows the EXAFS spectra, the best fits, and the corresponding Fourier transforms of wild-type and mutant sulfite oxidase. The Fourier transforms are significantly different, but both suggest that the EXAFS is dominated by two major interactions, giving rise to the transform peaks at $R + \Delta \approx 1.8$ and 2.3 Å, which are attributable to Mo═O and Mo−S interactions, respectively.[8] The Mo═O transform peak is more intense in the mutant, while for the Mo−S peak the reverse is true, with the wild-type protein having the larger Mo−S peak. Quantitative analysis by EXAFS curve-fitting gives an overall best fit for the wild-type protein that is very similar to that obtained previously for the chicken liver enzyme[8] with two Mo═O at 1.71 Å and three Mo−S at 2.41 Å. For the C207S mutant, however, the overall best fit indicates the presence of three Mo═O ligands at 1.74 Å and two Mo−S ligands at 2.47 Å. The results of selected EXAFS curve-fitting analyses are summarized in Table 1. Figure 4 shows search profiles for the Mo═O and Mo−S coordination number for which other parameters (*i.e.* bond lengths and Debye−Waller factors) were freely floated and the resulting goodness of fit plotted against coordination number. For the Mo═O profile, well-defined minima are observed at coordination number values of about 2 and 3 for the wild-type enzyme and the C207S mutant, respectively. Conversely, the Mo−S profile indicates coordination numbers of 3 and 2 for the wild-type protein and the C207S mutant, respectively.

Figure 5 shows search profiles for molybdenum−oxygen bond lengths in the C207S mutant enzyme. A metal coordination of two indistinguishable short oxygen (Mo═O) ligands, and two sulfur (Mo−S) ligands was assumed, and a third oxygen ligand was added. The bond length of the additional oxygen was systematically varied, and the Debye−Waller factors of both types of molybdenum−oxygen ligands were constrained in the fit to be equal. As with Figure 4, parameters were refined for every point in the search profile. A clear minimum for the additional molybdenum oxygen bond length occurs between

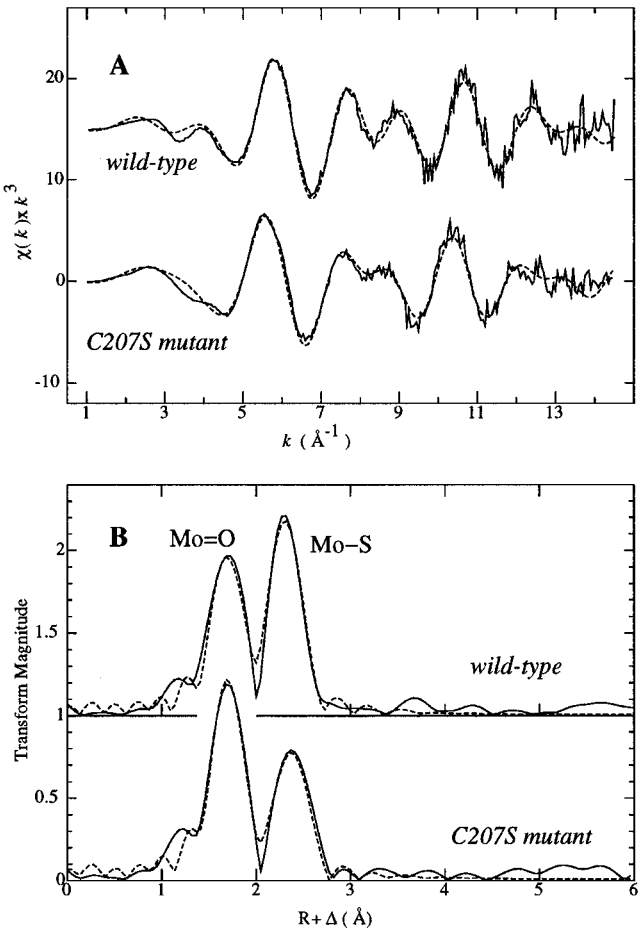

Figure 3. Molybdenum K-edge EXAFS of C207S mutant and wild-type sulfite oxidase. The solid lines show experimental data, while the broken lines show the best fit (Table 1). Part A shows the EXAFS oscillations and part B shows the corresponding Fourier transforms, phase-corrected for Mo−O backscattering.

1.67 and 1.80 Å, which is the range for a terminal Mo═O ligand. The bond lengths of the other two Mo═O ligands vary systematically across the minimum, maintaining an average

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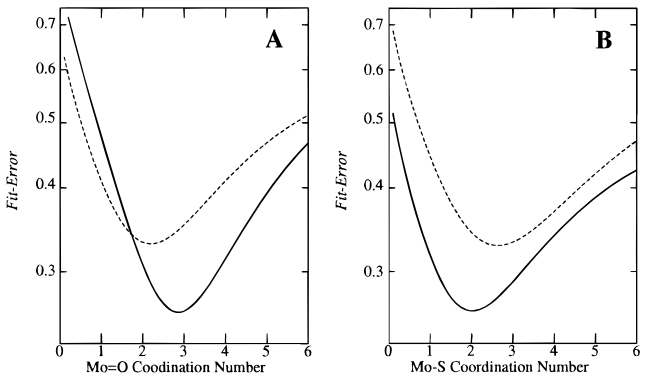

Figure 4. Search profiles for M═O (A) and Mo−S (B) coordination numbers. The solid lines indicate the profiles for the C207S mutant, and the broken lines those for the wild-tye protein. Search profiles were calculated by refining all parameters except for coordination numbers, which were varied systematically over the range shown on the abscissa of the plots. For the Mo═O profiles the coordination numbers for Mo−S were fixed at 2 and 3 for mutant and wild-type, respectively. For the Mo−S profiles, Mo═O coordination numbers were 3 and 2 for mutant and wild-type, respectively. The ordinate of the plots (the fit-error) is defined in Table 1 and is plotted on a log scale.

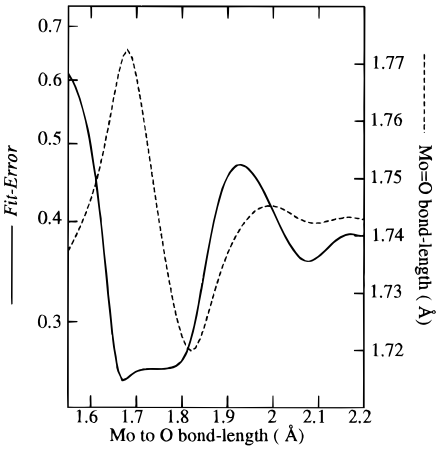

Figure 5. Molybdenum-to-oxygen bond length search profiles. Search profiles were calculated for a molybdenum coordination of two Mo═O and two Mo−S ligands, plus an additional oxygen. The Debye−Waller factors of both sets of oxygen ligands were constrained to be equal in the refinements and the bond length of the additional oxygen was systematically varied (the abscissa of the figure). The solid line shows the variation of the fit-error (plotted on a log scale, and defined in Table 1), and the broken line shows the variation of the length of the two other Mo═O bonds as a function of the molybdenum-to-oxygen bond length of the additional oxygen.

value of approximately 1.74 Å. This average value corresponds to the best fit with three Mo═O ligands (see Table 1).

Interestingly, Figure 5 shows a small minimum for the third Mo═O bond length at 1.69 Å, and at this minimum the value obtained for the common Mo═O Debye−Waller factor σ^2 is 0.0014 Å^2 (see Table 1). This value is very close to the ideal value expected for an isolated Mo═O ligand,[22] suggesting the presence of one short (1.67 Å) and two longer (1.77 Å) Mo═O ligands. However, we also note that this difference in Mo═O bond lengths is very close to the resolution of the EXAFS data (approximately given by $\Delta R = \pi/2k$), and thus that to conclude that two different Mo═O bond lengths are present might be bordering on the information content of the data.

Figure 5 also shows that longer molybdenum-to-oxygen bond lengths, such as would be expected from a Mo−O(Serine-207) coordination (*i.e.* 1.9−2.2 Å), give significantly a poorer fit than bond lengths in the distance range from a tri-oxo site. Overall, Figure 5 clearly indicates that our EXAFS data suggest a tri-oxo active site, and do not support a Mo−O(Serine-207) coordination.

Tri-oxo coordination is an unusual structural motif in molybdenum chemistry,[23] and its presence in the C207S mutant is quite unexpected.[24] The numbers of directly coordinated ligands estimated from EXAFS curve-fitting are generally accepted to have an accuracy of approximately ±25%, and thus conclusions based on this alone would be somewhat tenuous. However, the accuracy for bond length determination by EXAFS is considerably better, with a typical absolute accuracy of $<\pm 0.02$ Å. A considerably better *relative* accuracy is expected (perhaps by a factor of 10) when similar species are compared, as is the case in the present work. Our curve-fitting analysis indicates significant differences between wild-type and mutant enzymes for both Mo═O and Mo−S bond lengths. The Mo═O bond lengths are increased from 1.71 Å for the wild-type protin to 1.74 Å for the mutant, and the Mo−S bond lengths are also increased from 2.41 (wild-type) to 2.47 Å (mutant). Mayer's extensive survey[25] of model compounds indicates that Mo═O bond lengths systematically increase from 1.67 Å for mono-oxo species, to 1.70 Å for di-oxo species, to 1.73 Å for tri-oxo species. The observed bond lengths in the wild-type and C207S mutant proteins are thus quantitatively consistent with a di-oxo site for the wild-type and a tri-oxo site for the C207S mutant. Additionally, the mean Mo−S bond length is also expected to be longer in a tri-oxo site due to the *trans* effect, since all possible coordination sites for Mo−S ligands are *trans* to a Mo═O ligand.

The above conclusions are also supported by bond-valence-sum calculations,[26,27] which were performed as previously described.[28] For best-fit of the wild-type enzyme (Table 1) we obtain a bond-valence-sum value V of 5.9, in excellent agreement with the formal Mo(VI) oxidation state of the metal, and well within the accepted uncertainty of ±0.25 for V.[27] The best fit of the mutant gives $V = 6.0$ (Table 1), which is also in excellent agreement with the formal metal oxidation state. Alternative fits, on the other hand, give unacceptable values for V (see Table 1).

Sulfur K-edge Near-Edge Spectra. Figure 6 shows the sulfur K-edge spectra of wild-type and C207S mutant sulfite oxidase, respectively. As expected, both spectra are dominated by contributions from the protein methionine residues (and to a lesser extent from protein cysteine),[29] and no apparent metal−thiolate components are visible in the spectra. If we assume that the sulfur K-edge near-edge spectra of the molybdenum-

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(24) We also considered the possibility that the mutant protein might be unstable, perhaps losing molybdenum as molybdate during the preparation of concentrated EXAFS samples. If this were the case then an apparent increase in Mo═O coordination number might be observed due to molybdate contamination. In an attempt to eliminate this possibility, a sample of the mutant enzyme was rapidly gel-filtered on a Sephadex G100 column, with the eluant flowing directly into an EXAFS sample cuvette, which was then frozen and the XAS measured immediately. The Mo K-edge near-edge spectrum of enzyme thus treated was found to be identical to that shown in Figure 2, and we conclude that there is no significant contamination with low molecular weight species. The Mo K-edge XAS spectrum of the mutant protein also proved to be totally reproducible from preparation to preparation.

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(29) Human sulfite oxidase contains five methionine residues and six cysteine residues.[10]

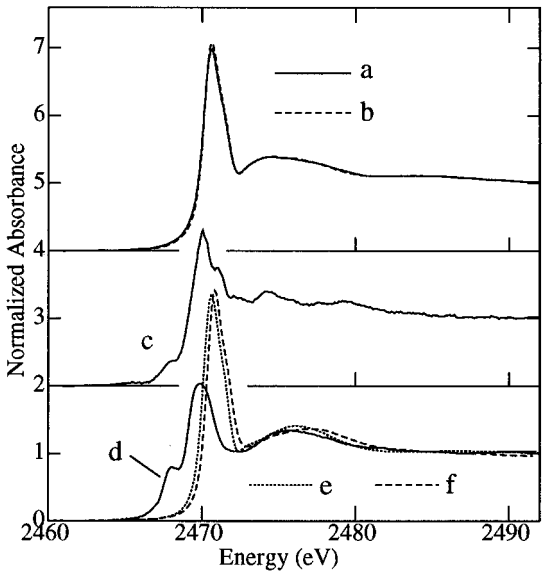

Figure 6. Sulfur K-edge near-edge spectra of C207S mutant (a) and wild-type (b) sulfite oxidase. Trace c is that obtained by subtracting 0.92 of trace a from trace b. Traces d, e, and f show the near-edge spectra of [tris(3,5-dimethylpyrazole) borate)]MoO[SC_2H_4O], cysteine, and methionine, respectively.

cofactor dithiolene is very similar in the two samples, then the spectra should differ only by the contribution of cysteine 207 in the wild-type protein.[30] Figure 6c shows the difference spectrum resulting from subtraction of the calculated fraction (based on the known sulfur content of the two proteins) of spectrum 6b from spectrum 6a. Considerable care was taken to ensure accurate normalization of the data (see the Experimental Section) upon which the validity of the difference spectrum depends. Figure 6c appears to be a normal near-edge spectrum, and does not contain any inverted features typical of inappropriately generated difference spectra. Nevertheless, the features of the difference spectrum that are close to or higher in energy than the methionine-dominated protein spectrum will be more subject to artifacts of the difference procedure than features of lower energy, and we therefore restrict our discussion to the latter.

The spectrum is shifted to lower energy with respect to that of cysteine and methionine (see Figures 6e and 6f), with a rising edge at about 2469.4 eV and a small but distinct pre-edge feature at about 2468.0 eV. Figure 6d compares the sulfur K-edge near-edge spectrum of a Mo(V) complex containing a single aliphatic thiolate ligand. The small peak at 2468.0 eV is typical of sulfur K-edge spectra of molybdenum thiolate complexes,[31] and similar features are observed in many transition metal thiolate complexes.[32] Such features are thought to arise from 1s → 3p transition with the sulfur 3p vacancies arising from the covalency of the metal−sulfur bond. The analogous pre-edge transitions are particularly well understood for copper chloride complexes.[21,32,33] Potentially, the intensity and position of the 2468.0-eV feature provides a direct probe of ligand covalency,[21,32,34] although further studies of model compounds are needed for a quantitative analysis.[35]

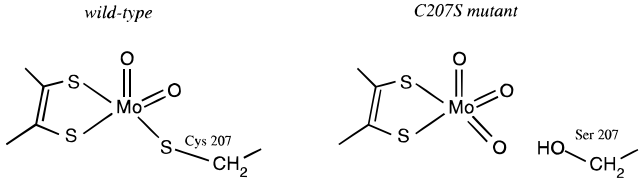

Figure 7. Proposed structures for the molybdenum-active sites of wild-type and C207S mutant sulfite oxidases. While geometric information is not available from the present EXAFS analysis, the oxo groups are expected to be *cis* [*e.g.* see ref 23], and are therefore shown as such.

Conclusions

In summary, the data presented here indicate that the active site of the C207S mutant sulfite oxidase is a novel tri-oxo molybdenum species with two thiolate ligands. Wild-type human sulfite oxidase possesses a di-oxo molybdenum site with three thiolate ligands. In conjunction with the results of the *in vitro* mutation studies[10] our EXAFS results indicate that cysteine-207 is a ligand of molybdenum in wild-type human sulfite oxidase. The two additional sulfur ligands almost certainly originate from the dithiolene group of the molybdenum cofactor. This conclusion is strengthened by the X-ray crystallography of *D. gigas* aldehydeoxidoreductase,[6] *Rhodobacter sphaeroides* dimethylsulfoxide reductase,[35] and *Pyrococcus furiosus* aldehydeoxidoreductase,[36] indicating coordination of the cofactor dithiolene(s) to molybdenum or tungsten at the active sites of these proteins. It seems likely that coordination of Mo or W to dithiolene sulfur is a universal feature of molybdoenzymes, and may in fact be *sine qua non* for the binding of the metal to the host proteins. Proposed structures for the active sites of the wild-type and mutant proteins are shown in Figure 7.

The finding that serine-207 apparently does not bind to molybdenum in place of the cysteine was unexpected. Possibly the slightly shorter bond lengths of the serine are insufficient to allow coordination to the molybdenum. Further experiments with additional site-directed mutants of cysteine 207 are planned to address this issue.

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(30) We note that this assumption is unlikely to be rigorously true as the Mo−S bond lengths are somewhat longer in the mutant. We also note that while some caution is justified in interpreting the features of the difference spectrum in Figure 6, the small changes in the X-ray absorption spectrum giving rise to the difference spectrum are of about the same size as a typical EXAFS oscillation at about $k \approx 9$ Å^{-1}, and thus, given accurate normalization, should be of equivalent reliability.

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