Inorg. Chem. **2006**, *45*, 7488–7492

Inorganic Chemistry
Article

Structure of the Active Site of Sulfite Dehydrogenase from *Starkeya novella*

Christian J. Doonan,† Ulrike Kappler,‡ and Graham N. George*,†

Department of Geological Sciences, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5E2, Canada, and the Centre for Metals in Biology, School of Molecular & Microbial Sciences, The University of Queensland, Brisbane, Queensland 4072, Australia

Received May 10, 2006

In this paper, we report the results of molybdenum K-edge X-ray absorption studies performed on the oxidized and reduced active sites of the sulfite dehydrogenase from *Starkeya novella.* Our results provide the first direct structural information on the active site of the oxidized form of this enzyme and confirm the conclusions derived from protein crystallography that the molybdenum coordination is analogous to that of the sulfite oxidases. The molybdenum atom of the oxidized enzyme is bound by two Mo═O ligands at 1.73 Å and three thiolate Mo–S ligands at 2.42 Å, whereas the reduced enzyme has one oxo at 1.74 Å, one long oxygen at 2.19 Å (characteristic of Mo–OH_2), and three Mo–S ligands at 2.40 Å.

Introduction

Sulfite oxidases catalyze the two-electron oxidation of sulfite to sulfate

$$SO_3^{2-} + H_2O \rightarrow SO_4^{2-} + 2H^+ + 2e^-$$

They have been identified in mammals, insects, plants, and a variety of prokaryotic microorganisms.[1−4] In animals, the enzyme is localized in the mitochondrial intermembrane space and functions as the final step in the catabolism of sulfur-containing amino acids. In plants, sulfite oxidase is localized in the peroxisome and functions in either sulfite detoxification or regulation of the sulfate pool.[6] Sulfite oxidases are thus widespread in nature, and to date, all reported enzymes of this type contain molybdenum at their active site. Molybdenum is found at the active site of a family of enzymes, with the notable exception of nitrogenase,[7] in association with a novel pyranopterindithiolene cofactor, known as molybdopterin or the molybdenum cofactor.[8,9] The molybdenum is ligated by the dithiolene moiety of the cofactor,[10] with either one or two cofactors being bound at the active site, depending on the enzyme.[11] The coordination

* To whom correspondence should be addressed. Phone: 306-966-5722. Fax: 306-966-8593. E-mail: g.george@usask.ca.
† University of Saskatchewan.
‡ University of Queensland.

(1) Rajagopalan, K. V. In *Molybdenum and Molybdenum-Containing Enzymes*; Coughlan, M. P., Ed.; Pergamon Press: Oxford, U. K., 1980; pp 243−272.
(2) Braaten, A. C.; Bentley, M. M. *Biochem. Genetics* **1993**, *31*, 375−91.
(3) Eilers, T.; Schwarz, G.; Brinkmann, H.; Witt, C.; Richter, T.; Nieder, J.; Koch, B.; Hille, R.; Hänsch, R.; Mendel, R. R. *J. Biol. Chem.* **2001**, *276*, 46989−46994. (b) Hille, R. *Structure* **2003**, *11*, 1189−1197. (c) Schrader, N.; Fischer, K.; Theis, K.; Mendel, R. R.; Schwartz, G.; Kisker, K. *Structure* **2003**, *11*, 1251−1263.
(4) Kappler, U.; Bennett, B.; Rethmeier, J.; Schwarz, G.; Deutzmann, R.; McEwan, A. G.; Dahl, C. *J. Biol. Chem.* **2000**, *275*, 13202−13212.
(5) D'Errico, G.; Di Salle, A.; La Cara, F.; Rossi, M.; Cannio, R. *J. Bacteriol.* **2006**, *188*, 694−701.
(6) Hänsch, R.; Mendel, R. *Photosynthesis Res.* **2005**, *86*, 337−343.
(7) Rees, D. C.; Tezcan, A.; Haynes, C. A.; Walton, M. Y.; Andrade, S.; Einsle, O.; Howard, J. B. *Philos. Trans. R. Soc. A.* **2005**, *363*, 971−984.
(8) Rajagopalan, K. V. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1991**, *64*, 215−290. (b) Rajagopalan, K. V.; Johnson, J. L. *J. Biol. Chem.* **1992**, *267*, 10199−10202.
(9) Molybdenum enzymes have previously been described as all involving two-electron redox chemistry at molybdenum, coupled with the transfer of an oxygen atom. While this is true for the majority of molybdenum enzymes (and for their close relatives the tungsten enzymes), there are some exceptions. The tungsten enzyme acetylene hydratase (Rosner, B. M.; Schink, B. *J. Bacteriol.* **1995**, *177*, 5767−5772) catalyzes a net hydration reaction rather than a redox one, and formate oxidation to CO_2 by *Eschericia coli* formate dehydrogenase H does not involve oxygen atom transfer (Khangulov, S. V.; Gladyshev, V. N.; Dismukes, G. C.; Stadtman, T. C. *Biochemistry*, **1998**, *37*, 3518−3528). Furthermore, the presence of the potentially redox-active selenosulfide at the active site (George, G. N.; Colangelo, C. M.; Dong, J.; Scott, R. A.; Khangulov, S. V.; Gladyshev, V. N.; Stadtman, T. C. *J. Am. Chem. Soc.* **1998**, *120*, 1267−1273), suggests that the molybdenum in formate dehydrogenases might not be redox active during the catalysis (George, G. N.; Costa, C.; Moura, J. J. G.; Moura, I. *J. Am. Chem. Soc.* **1999**, *121*, 2625−2626).
(10) Kisker, C.; Schindelin, H.; Pacheco, A.; Wehbi, W. A.; Garrett, R. M.; Rajagopalan, K. V.; Enemark, J. E.; Rees, D. C. *Cell* **1997**, *91*, 1−20. (b) Karakas, E.; Wilson, H. L.; Graf, T. N.; Xiang, S.; Jaramillo-Busquets, S.; Rajagopalan K. V.; Kisker, C. *J. Biol. Chem.* **2005**, *280*, 33506−33515.

10.1021/ic0607944 CCC: $33.50
© 2006 American Chemical Society
Published on Web 08/03/2006

of the molybdenum in sulfite oxidases[10,12] and the closely related assimilatory nitrate reductases[13,14] contains the dithiolene of a single cofactor, two terminal oxo groups, and one cysteine ligand from the polypeptide backbone.

Recently a sulfite dehydrogenase (SDH) from the facultatively chemolithotrophic, sulfur-oxidizing soil bacterium *Starkeya novella* has been characterized by protein crystallography.[15] Unlike the plant sulfite oxidase, sulfite dehydrogenases do not transfer electrons to molecular oxygen at an appreciable rate. The crystallographic study of the bacterial SDH indicates a similar molybdenum coordination to that established for assimilatory nitrate reductase[14] and chicken liver sulfite oxidase,[10] with one long (∼2.3 Å) and one short (∼1.7 Å) molybdenum−oxygen bond, and thiolate ligation from the dithiolene moiety of the molybdenum cofactor and from one cysteine (C104). In the case of sulfite oxidase there was a discrepancy between active site structures elucidated by X-ray crystallography and X-ray absorption spectroscopy. This difference was attributed to X-ray photoreduction of the enzyme during crystallographic data acquisition.[16] Samples of SDH, initially in oxidized and reduced forms were examined crystallographically, but only a reduced active site was observed for both, presumably for the same reason (i.e., X-ray photoreduction).[15] Thus, to date, no direct information about the coordination of the oxidized molybdenum center in SDH is available. We report herein a structural study of the molybdenum active sites of both oxidized and reduced *S. novella* SDH using Mo K-edge X-ray absorption spectroscopy.

Materials and Methods

Samples. *S. novella* SDH (1 mM) was prepared as previously described,[17] transferred to (2 × 10 × 10 mm) Lucite sample cuvettes, frozen, and stored in liquid nitrogen until data acquisition. In all cases, 20 mM Tris buffer was used at pH 7.8. The as-isolated SDH enzyme contains a mixture of oxidized and reduced forms.[4,17] The fully oxidized enzyme was prepared by incubation on ice with a 2 mM ferricyanide solution (final concentration) for 5 min, followed by gel filtration (PD-10 column, GE Healthcare) to remove the ferricyanide and prevent loss of activity by the inhibitory effects of ferricyanide. The reduced enzyme was generated by treating oxidized SDH with an excess (10 mM) of sodium dithionite solution for 1 min with a trace (1 μM) of methyl viologen. Mo(V) EPR spectroscopy was used to monitor the extent of reduction of the molybdenum center (residual Mo(V)), double integration using a 1mM Tp*$MoOCl_2$ standard,[18] and the residual Mo(V) content was found to be between 10 and 15%.

XAS Data Collection. XAS measurements were conducted at the Stanford Synchrotron Radiation Laboratory with the SPEAR 3 storage ring containing between 90 and 100 mA at 3.0 GeV. Molybdenum K-edge data were collected on the structural molecular biology XAS beamline 9-3 with a wiggler field of 2 T, employing a Si(220) double-crystal monochromator. 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-filled ionization chambers, and X-ray absorption was measured as the Mo Kα fluorescence excitation spectrum using an array of 30 germanium detectors.[19] During data collection, the samples were maintained at a temperature of approximately 10 K using an Oxford instruments liquid helium flow cryostat. For each sample, eight to twelve scans were accumulated, and the energy was calibrated by reference to the absorption of a molybdenum metal 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.

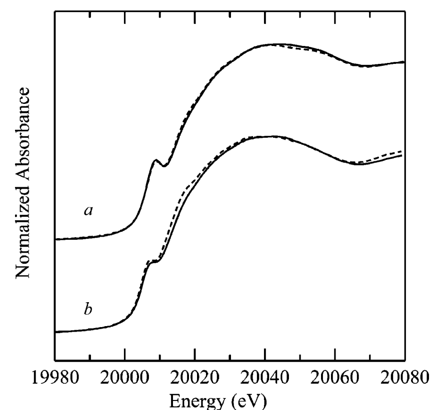

Figure 1. Comparison of Mo K near-edge spectra of *S. novella* sulfite dehydrogenase (solid lines) and human sulfite oxidase (broken lines) in oxidized (a) and reduced (b) forms.

XAS Data Analysis. The EXAFS oscillations, $\chi(k)$, were quantitatively analyzed by curve-fitting using the EXAFSPAK suite of computer programs,[20] as described by George et al.,[21] using ab initio theoretical phase and amplitude functions calculated using FEFF, version 7.2.[22] No smoothing, filtering, or related operations were performed on the data.

EPR Spectroscopy. Electron paramagnetic resonance (EPR) spectra were collected at a sample temperature of 80 K using a Bruker EMX EPR spectrometer. Data reduction and spectral simulations were performed as described by George et al.[23]

Results and Discussion

Mo K Near-Edge Analysis. Figure 1 compares the X-ray absorption near-edge spectra of *S. novella* sulfite dehydrogenase (SDH) and wild-type human sulfite oxidase (SOX).

(11) Hille, R. *Chem. Rev.* **1996**, *96*, 2757−2816.
(12) Harris, H. H.; George, G. N.; Rajagopalan, K. V. *Inorg. Chem.* **2006**, *45*, 493−495.
(13) George, G. N.; Mertens, J. A.; Campbell, W. A. *J. Am. Chem. Soc.* **1999**, *121*, 9730−9731.
(14) Fischer, K.; Barbier, G.; Hecht, H.-J.; Mendel, R. R.; Campbell, W. H.; Schwarz, G. *Plant Cell* **2005**, *17*, 1167−1179.
(15) Kappler, U.; Bailey, S. *J. Biol. Chem.* **2005**, *280*, 24999−25007.
(16) George, G. N.; Pickering, I. J.; Kisker, C. *Inorg. Chem.* **2005**, *38*, 2539−2540.
(17) Kappler, U.; McEwan, A. G. *FEBS Lett.* **2002**, *529*, 208−214.
(18) Tp* = hydrotris(3,5-dimethylpyrazolyl)borate; Cleland, W. E., Jr.; Barnhart, K. M.; Yamanouchi, K.; Collison, D.; Mabbs, F. E.; Ortega, R. B.; Enemark, J. H. *Inorg. Chem.* **1987**, *26*, 1017−1025.
(19) Cramer, S. P.; Tench, O.; Yocum, M.; George, G. N. *Nucl. Instrum. Methods Phys. Res.* **1988**, *A266*, 586−591.
(20) The EXAFSPAK program suite was developed by one of the authors (G.N.G.) and is available from http://ssrl.slac.stanford.edu/exafspak.html.
(21) George, G. N.; Garrett, R. M.; Prince, R. C.; Rajagopalan, K. V. *J. Am. Chem. Soc.* **1996**, *118*, 8588−8592.
(22) Rehr, J. J.; Mustre de Leon, J.; Zabinsky, S. I.; Albers, R. C. *J. Am. Chem. Soc.* **1991**, *113*, 5135−5140. (b) Mustre de Leon, J.; Rehr, J. J.; Zabinsky, S. I.; Albers, R. C. *Phys. Rev.* **1991**, *B44*, 4146−4156.
(23) George, G. N.; Prince, R. C.; Kipke, C. A.; Sunde., R. A.; Enemark, J. E. *Biochem. J.* **1988**, *256*, 307−309.

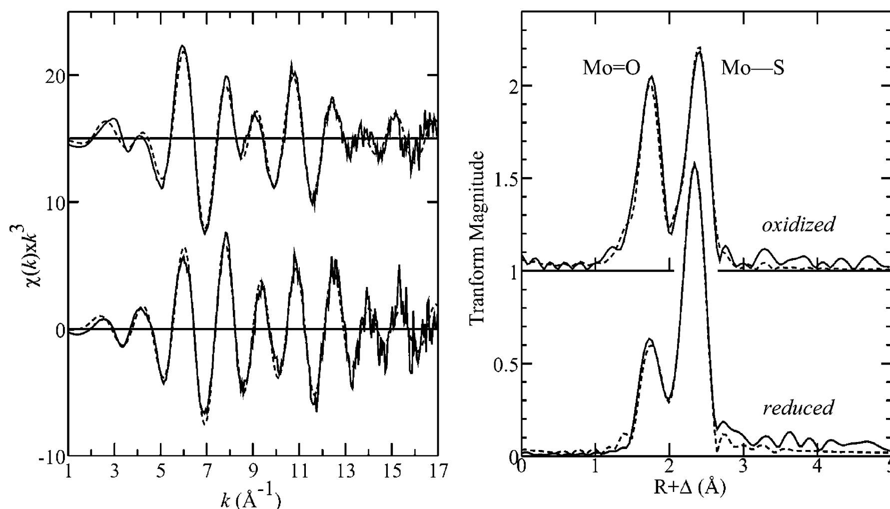

Figure 2. EXAFS and Mo−S phase-corrected EXAFS Fourier transforms of *S. novella* sulfite dehydrogenase in oxidized (a) and reduced (b) forms. The solid lines show the experimental data, while the broken lines indicate the best fit using the parameters given in Table 1.

The spectra of the oxidized enzymes (shown in Figure 1a) are very similar, indicating that the oxidized active site geometries of the two enzymes are essentially identical. Both spectra show a preedge feature at approximately 20 009.0 eV resulting from a Mo 1s → (Mo═O)π* transition.[24] Previous work has established that the intensity of this feature is correlated to the number of terminal oxygen ligands bound to Mo.[25−27] Thus, the present data indicates that the oxidized active site of *S. novella* sulfite dehydrogenase contains a MoO_2 moiety analogous to wild-type human sulfite oxidase.[12,21,25] The near-edge regions of the two enzymes are compared in Figure 1. The minor differences observed may be attributed to subtle geometric and electronic structure differences at the respective active sites. Nevertheless, the similarity of the near-edge spectra shown in Figure 1 indicates considerable similarity between the fully oxidized active sites of SOX and SDH. The spectra of reduced enzymes show more significant, but still subtle, differences. Both spectra show nearly identical preedge features at approximately 20 009 eV; however, sulfite oxidase exhibits a small edge shift to lower energy. This may be explained by the residual Mo(V) in SDH, estimated to be between 10 and 15% from EPR analysis. The active sites of SDH and SOX are already known to differ because only the putative high-pH signal is detectible for SDH, whereas characteristic low-pH and high-pH signals are known for SOX.[4,28]

Mo K-Edge EXAFS. Figure 2 shows the EXAFS and EXAFS Fourier transforms of oxidized and reduced SDH, together with the best fits from the curve-fitting analysis. The EXAFS Fourier transforms for both the oxidized and reduced enzyme display two major features at approximately 1.7 and 2.3 Å, attributable to Mo═O and, primarily, Mo−S bonds, respectively. For the oxidized enzyme, the size of the two Fourier transform peaks are analogous; however, for the reduced form of the enzyme, the peak assigned to the Mo═O moiety is significantly less intense. Quantitative curve-fitting analysis indicates that the oxidized active site of SDH possesses three Mo−S at 2.42 Å and two Mo═O units at 1.73 Å, while the reduced enzyme shows three Mo−S interactions at 2.40 Å, one Mo−O at 2.19 Å, and one Mo═O at 1.74 Å. Overall, these results are very similar to those of human sulfite oxidase.[12] Oxidized sulfite oxidase shows essentially identical (within the accuracy)[29] Mo−S and Mo═O distances of 2.42 and 1.73 Å, respectively. Again, the parameters of the reduced enzyme are a little different from those of sulfite oxidase, which shows three Mo−S at 2.37 Å, one Mo−O at 2.30 Å, and one Mo═O at 1.72 Å. The presence of residual Mo(V) in the reduced sulfite dehydrogenase sample will obviously increase the uncertainty in these values, and this might explain why the Mo−S bond lengths are slightly longer than those for sulfite oxidase. This correspondence of bond lengths further reinforces the conclusion the overall structures of the molybdenum active

Table 1. EXAFS Curve-Fitting Results for the Oxidized Active Site of *S. novella* Sulfite Dehydrogenase[a]

sample	interaction	N	R (Å)	σ^2 (Å^2)	ΔE_0 (eV)	F
oxidized	Mo═O	2	1.728(2)	0.0029(1)	−10(1)	0.239
	Mo−S	3	2.421(2)	0.0044(1)		
reduced	Mo═O	1	1.743(3)	0.0017(3)	−11(1)	0.329
	Mo−O	1	2.193(11)	0.0026(9)		
	Mo−S	3	2.395(3)	0.0029(1)		

[a] Coordination numbers, N; interatomic distances, R, given in Å; Debye−Waller factors, σ^2 (the mean-square deviations in interatomic distance), in Å^2; and the threshold energy shifts, ΔE_0, given in eV. The values in parentheses are the estimated standard deviations obtained from the diagonal elements of the covariance matrix. The fit-error function F is defined as $\sqrt{\Sigma k^6(\chi(k)_{calcd}-\chi(k)_{exptl})^2/\Sigma k^6\chi(k)_{exptl}{}^2}$; the summation is over all data points included in the refinement.

(24) Kutzler, F. W.; Natoli, C. R.; Misemer, D. K.; Donaich, S.; Hodgson, K. O. *J. Chem. Phys.* **1980**, *73*, 3274−3288.
(25) Cramer, S. P.; Wahl, R.; Rajagopolan, K. V. *J. Am. Chem. Soc*. **1981**, *103*, 7721−7727.
(26) George, G. N.; Hilton, J.; Temple, C.; Prince, R. C.; Rajagopolan, K. V. *J. Am. Chem. Soc.* **1999**, *121*, 1256−1266.
(27) Kutzler, F. W.; Scott, R. A.; Berg, J. M.; Hodgson. K. O.; Donaich, S.; Cramer, S. P.; Chang, C. H. *J. Am. Chem. Soc.* **1981**, *103*, 6083−6088.
(28) Raitsimring, A. M.; Kappler, U.; Feng, C.; Astashkin, A. V.; Enemark, J. H. *Inorg. Chem.* **2005**, *44*, 7283−7285.

(29) The generally accepted accuracy for EXAFS bond-length determination is ±0.02 Å. The relative accuracy between two similar samples (e.g., oxidized and reduced enzyme) is likely to be rather better than this.

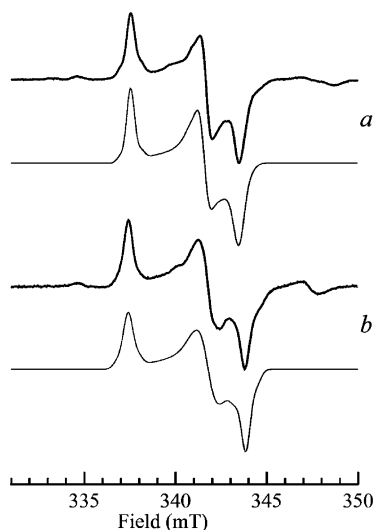

Figure 3. Mo(V) EPR spectrum of *S. novella* sulfite dehydrogenase compared with the high-pH signal of human sulfite oxidase (data from ref 30). The spectra are aligned to a common microwave frequency of 9.3877 GHz. Bold lines show experimental spectra, while the solid lines show computer simulations which were calculated using g values of $g_{(x,y,z)}$ = 1.9508, 1.9626, and 1.9879 for sulfite dehydrogenase and $g_{(x,y,z)}$ = 1.9529, 1.9636, and 1.9871 for sulfite oxidase. For sulfite dehydrogenase, the pH was 7.8 (20 mM Tris buffer), and for sulfite oxidase the pH was 9.0 (50 mM bis-tris-propane buffer).

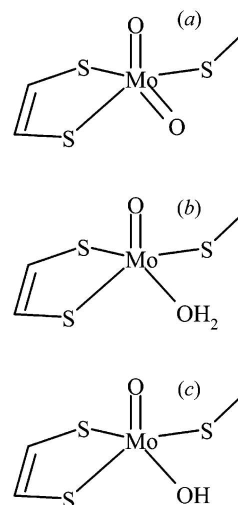

Figure 4. Postulated molybdenum active site structures for fully oxidized Mo(VI) (a), fully reduced Mo(IV) (b), and Mo(V) (c) sulfite dehydrogenases.

sites of the two enzymes are very similar, despite the known differences in Mo(V) EPR and reactivity. Two of the three Mo−S interactions observed are expected to come from the sulfurs of the dithiolene moiety of the molybdopterin cofactor, and the third sulfur contribution to the EXAFS results from bound cysteine (C104), as observed in the crystal structure. These EXAFS results demonstrate that the oxidized form of the active site of sulfite dehydrogenase is essentially identical to wild-type human and chicken sulfite oxidase. The bond lengths for the reduced SDH are in reasonable agreement with those obtained from protein crystallography. However, a small discrepancy is observed for between the distances of the long Mo−O interaction found in the EXAFS analysis (2.19 Å) and the crystallographic studies (2.3 Å). This nonconformity is consistent with the larger uncertainties expected from crystallography. The EXAFS-derived bond length definitively identifies this oxygen as a water ligand (Mo−OH_2), rather than a hydroxide (Mo−OH). A similar conclusion has been reported for sulfite oxidase.[12]

Our Mo(V) EPR spectra (Figure 3) confirmed earlier results[28] which indicated that SDH gave only the so-called high-pH EPR signal. This form of the enzyme is thought to correspond to a molybdenum coordination site free of direct coordination by anions,[31] and thus, the reduced EXAFS data set probably corresponds to predominantly (85−90%) Mo(IV) with no directly coordinated anions. The EPR g values of the SDH high-pH signal are very similar to those of sulfite oxidase (from both chicken and human), and the so-called proton spin−flip lines arising from a strongly coupled exchangeable proton (also detected by multifrequency pulsed EPR[28]) are clearly seen.[32,33]

Mammalian sulfite oxidase and bacterial sulfite dehydrogenase have different properties. The sulfite oxidase crystal structure shows that the heme is located in a separate domain from the molybdenum,[10] and the enzyme requires a domain movement to function, but with sulfite dehydrogenase, on the other hand, the redox centers are locked in place.[15] Sulfite oxidase has an active site pocket about 5 Å from Mo with three arginine residues that can hold an anion such as sulfite adjacent to Mo,[10] but sulfite dehydrogenase lacks one of these three arginine residues, and this may be important in explaining the different EPR properties of the two enzymes. Despite this difference, sulfite dehydrogenase has higher turnover. The work we have presented here shows that there are considerable similarities between the molybdenum coordination environments of SO and SDH, and Figure 4 shows proposed structures for the active sites of all three oxidation states (we note that essentially identical structures have been proposed for sulfite oxidase). In particular, the structures of the molybdenum sites of oxidized sulfite oxidase and sulfite dehydrogenase are identical within the accuracy limits of our analyses. We conclude that the basis for the differences in enzyme activities does not have its roots in substantial differences in the first coordination sphere of the molybdenum active sites.

Acknowledgment. Parts of this work were carried out at the Stanford Synchrotron Radiation Laboratory (SSRL), a national user facility operated by Stanford University on behalf of the U.S. Department of Energy, Office of Basic Energy Sciences. The SSRL Structural Molecular Biology Program is supported by the Department of Energy, Office of Biological and Environmental Research, and by the National Institutes of Health, National Center for Research

(30) George, G. N.; Garret, R. M.; Prince, R. C.; Rajagopalan, K. V. *Inorg. Chem.* **2004**, *43*, 8456−8460.
(31) George, G. N.; Garrett, R. M.; Graf, T.; Prince, R. C.; Rajagopalan, K. V. *J. Am. Chem. Soc.* **1998**, *120*, 4522−4523.
(32) These are formally forbidden $\Delta m_I = \pm 1$ transitions resulting from a strongly coupled exchangeable proton in which $\mathbf{A}(^1H)$ is close to the magic angle in relation to **g**.
(33) George, G. N. *J. Magn. Reson.* **1985**, *64*, 384−394.

Resources, Biomedical Technology Program. Work at the University of Saskatchewan was supported by the Natural Sciences and Engineering Research Council (Award 283315), a Canada Research Chair award (G.N.G.), the University of Saskatchewan, the Province of Saskatchewan, the National Institutes of Health (GM57375), the Canadian Institute for Health Research, and the Canada Foundation for Innovation (Award 201742). Work at the University of Queensland was supported by a grant and fellowship to U.K. by the University of Queensland.

IC0607944