X-ray Absorption Spectroscopy of Chicken Sulfite Oxidase Crystals

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Received December 21, 1998

Introduction

Sulfite oxidase catalyzes the physiologically vital oxidation of sulfite to sulfate.[1] Residing in the mitochondrial intermembrane space, the enzyme is dimeric with a subunit weight of about 52 000. Each monomer contains molybdenum associated with a single molybdopterin 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 reoxidation of the molybdenum, first to Mo^{V} and then to Mo^{VI}, by intramolecular electron transfer to the cytochrome b_5 site.[2] With the exception of nitrogenase, all molybdenum enzymes that have been described to date[3] contain a novel pterin cofactor in which the molybdenum is bound by the pyranodithiolene of the molybdopterin cofactor[3,4] (see Figure 1). Until recently, structural information on molybdenum enzymes was derived almost entirely from spectroscopy of the enzymes and of model compound systems.[3] As one of the most intensively studied molybdenum enzymes, sulfite oxidase can be regarded as the prototypical member of one class of molybdenum enzymes, those possessing di-oxo molybdenum sites when the enzyme is in the fully oxidized Mo^{VI} form.[5−8] Recently, the crystal structure of chicken sulfite oxidase has been reported at 1.9 Å resolution.[9] In contrast to the information available from previous X-ray absorption spectroscopic studies, the active site indicated by crystallography was a mono-oxo species (Figure 1). Because of this the possibility that the crystals did in fact contain a reduced molybdenum species was considered in the crystallographic work. We report herein an X-ray absorption spectroscopic study of polycrystalline sulfite oxidase prepared in the same manner as the previous single-crystal samples,[9] and compare this with data for frozen solutions of oxidized and reduced enzyme.

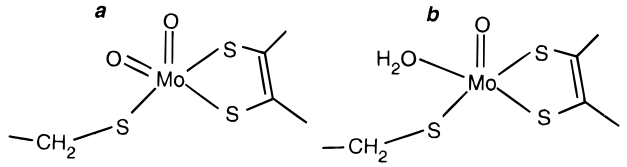

Figure 1. Schematic structures of the molybdenum active site of sulfite oxidase from (*a*) X-ray absorption spectroscopy of oxidized enzyme and from (*b*) crystallography. The coordinated water in (*b*) might alternatively be a hydroxyl.[9]

Experimental Section

Crystalline chicken sulfite oxidase was prepared as previously described.[9] A sample containing a large number of small crystals was mounted in a sealed quartz capillary and microscopically examined to ensure that no preferential crystal orientation was present and thus avoid polarization effects. X-ray absorption spectroscopy (XAS) was carried out at the Stanford Synchrotron Radiation Laboratory on beamline 7-3. X-ray absorption was measured as the X-ray Mo Kα fluorescence excitation spectrum with an array of thirteen germanium detectors,[10] and the sample was maintained at a temperature of approximately 10 K during data collection. Extended X-ray absorption fine structure (EXAFS) oscillations $\chi(k)$ were quantitatively analyzed with EXAFSPAK [http://ssrl.slac.stanford.edu/exafspak.html] using ab initio phase and amplitude functions generated with FEFF V7.02.[11] No smoothing, filtering, or related manipulation was performed upon the data.

XAS data on frozen solutions of human sulfite oxidase were collected as previously reported;[7,12] samples were prepared in a mixed buffer system consisting of 20 mM Tris, bis-Tris, and bis-Tris-propane pH 9.0, with no added chloride. Oxidized samples were prepared by air equilibration and reduced samples by addition of excess (10 mM final) sodium dithionite solution with 10 μM methyl viologen.

Results and Discussion

X-ray absorption near-edge spectra are in general very sensitive to electronic structure. Thus, the formal oxidation state of a sample can in principle be determined simply by examining the near-edge spectra. Figure 2 shows the near-edge spectrum and EXAFS Fourier transforms of polycrystalline sulfite oxidase compared with data for oxidized and reduced solutions of human sulfite oxidase. The near-edge spectrum of the crystalline sulfite oxidase sample is identical to that of the oxidized frozen solution sample, indicating the same geometry and oxidation state, and is distinctly different from that of the reduced frozen solution sample. Similarly, the EXAFS Fourier transforms of the

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10.1021/ic981451b CCC: $18.00
Published on Web 04/28/1999

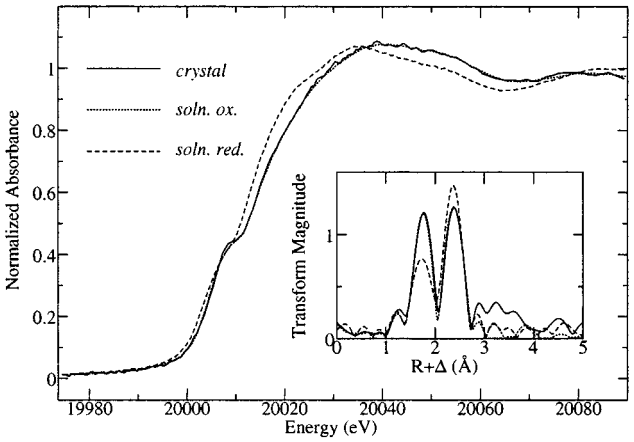

Figure 2. Mo K-edge X-ray absorption near-edge spectra of polycrystalline sulfite oxidase compared with oxidized (Mo^{VI}) and reduced (Mo^{IV}) frozen solution human sulfite oxidase. The inset shows the corresponding EXAFS Fourier transforms (k range 2.0−12.5 Å^{-1}, Mo−S phase-corrected).

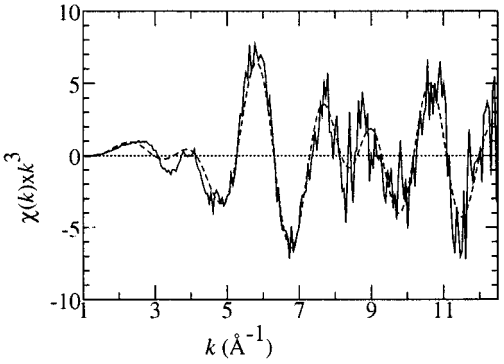

Figure 3. Mo K-edge EXAFS curve-fitting analysis of polycrystalline sulfite oxidase. The solid line shows the experimental data while the broken line shows the best fit obtained using procedures similar to those followed by George et al.[7] The parameters of the best fit were two Mo═O at 1.711(5) Å, $\sigma^2 = 0.0024(4)$ Å^2 and three Mo−S at 2.414(6) Å, $\sigma^2 = 0.0051(4)$ Å^2, $\Delta E_0 = -15(2)$ eV, consistent with the structure shown in Figure 1a. The values in parentheses are the estimated standard deviations (precisions) obtained from the diagonal elements of the covariance matrix and σ^2 are the Debye−Waller factors.

crystalline and oxidized solution sample are essentially identical, indicating the same metal coordination, while that of the reduced sample is quite distinct (we note that the structure above 3 Å in the EXAFS Fourier transform of the crystal data is primarily due to noise in this data set). EXAFS curve-fitting analysis of the crystalline sample is shown in Figure 3, and gives very similar results to those previously obtained for human[7] and for chicken[5,6] oxidized enzyme. The best fit indicates two Mo═O ligands at 1.71 Å, and three Mo−S at 2.41 Å, which can be compared with identical values obtained from the most recent solution study of human sulfite oxidase.[7]

These results unambiguously indicate that the crystals examined in the present study were in the oxidized, formally Mo^{VI}, oxidation state, with an active site structure similar to that of Figure 1a, and confirms that the oxidized chicken[5,6] and human enzymes have essentially identical active site structures.[7] The most probable source of the apparent discrepancy between crystallography and spectroscopy is photoreduction.[13] Because nearly identical preparations of crystals were used for the crystal structure[9] and the present work[14] we conclude that the crystals used by Kisker et al.[9] were initially oxidized. Chicken sulfite oxidase crystallizes with the monoclinic spacegroup $P2_1$, which, together with the high resolution of 1.9 Å, necessitates long data acquisition times and correspondingly long exposure to the X-ray beam. In addition, the crystallographic data acquisition employed about 2 orders of magnitude higher X-ray flux than did the XAS,[15] and used an X-ray energy with an approximately 6-fold greater sample X-ray absorption cross-section. Furthermore, the crystallography was conducted at the relatively high temperature of 95 K.[9] All of these factors will contribute toward X-ray photoreduction of the metal site, and it therefore seems very likely that photoreduction of the molybdenum site might have occurred in the crystallographic study, and that this is the cause of the apparent discrepancy between crystallography and spectroscopy. These findings may cast doubt upon the presumed redox status of metal sites in crystallographic studies of some other proteins, especially those for which long X-ray exposures have been used. The utility of simultaneous XAS and crystallographic measurements of proteins is currently being investigated at SSRL. These studies may provide not only an important indicator of the metal redox state and extent of photoreduction by the X-ray beam, but also independent metal bond-length restraints from analysis of the EXAFS.

Acknowledgment. SSRL is funded by DOE BES, with further support by DOE OBER and NIH. Research at Stony Brook was supported by a Targeted Research Opportunity Grant from SUNY Stony Brook. We thank K. V. Rajagopalan (Duke University) for commenting on this manuscript and J. Enemark (University of Arizona) for providing purified chicken sulfite oxidase.

Supporting Information Available: A detailed description of the X-ray absorption spectroscopy experimental procedure, plus a table of EXAFS curve-fitting results for polycrystalline sulfite oxidase. This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) See, for example: Rich, A. M.; Armstrong, R. S.; Ellis, P. J.; Lay, P. A. *J. Am. Chem. Soc.* **1998**, *120*, 10827−10836.

(14) In the crystallographic experiment crystals were transferred into mother liquor containing 20% glycerol cryoprotectant prior to data acquisition.[9] While glycerol was not added to either solution samples or crystals in the current experiments, we note from separate experiments on the enzyme in solution that sulfite oxidase is not reduced by glycerol, even after very long exposures. In fact, the enzyme is routinely stored in solution containing 50% glycerol at −20 °C, and no reduction is observed even after many months of storage.

(15) Crystallographic data were collected on SSRL beamline 7-1,[9] which uses a bent-triangular Si(111) focusing monochromator, while our XAS data were collected using SSRL beamline 7-3, which has no focusing optics and a correspondingly less intense X-ray beam. In addition, we used a Si(220) monochromator to obtain high energy resolution, which yields a correspondingly decreased X-ray intensity. Allowing for these factors, and for the relative distances from the source (the beamline 7 wiggler), we estimate a difference of at least 2 orders of magnitude in X-ray flux at the sample between these two beamlines.