Resonance Raman Characterization of the Molybdenum Center in Sulfite Oxidase: Identification of Mo=O Stretching Modes

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Received November 13, 1996

With the exception of nitrogenase, molybdenum is found in enzymes at the active site of a wide range of oxotransferases and is coordinated by one or two terminal oxo groups and the dithiolene unit of one or two molybdopterins (MPT).[1] The recent X-ray crystal structures of the aldehyde oxidoreductase (AOR) from *Desulfovibrio gigas*[2] and the DMSO reductases (DR) from *Rhodobacter sphaeroides*[3a] and *Rhodobacter capsulatus*[3b] have shown the diversity of the active site molybdenum coordination environments (Figure 1). On the basis of spectroscopic, primary sequence, and cyanide inhibition data, these enzymes are considered to be archetypical members of large families of enzymes: *D. gigas* AOR representing the xanthine oxidase family with a (MPT)$Mo^{VI}OS$ active site and no protein ligands to Mo, typified by xanthine oxidase and xanthine dehydrogenase; *R. sphaeroides* DR defining a family of molybdoproteins with a $(MPT)_2Mo^{VI}O$ active site and one protein ligand to Mo (serine, cysteine or selenocysteine), that includes dissimilatory nitrate reductases, formate dehydrogenases and biotin-S-oxide reductase. A third family, encompassing sulfite oxidases and assimilatory nitrate reductases, involving a (MPT)$Mo^{VI}O_2$ active site with one cysteinyl S ligand to Mo (C207 in rat and human sulfite oxidase), has been proposed on the basis of X-ray absorption,[4] EPR,[5] primary sequence,[6] mutagenesis,[7] and cyanide inhibition data, as shown in Figure 1. Here we report a resonance Raman (RR) study of the molybdenum domain of the wild-type and C207S mutant forms of recombinant human sulfite oxidase (hSO). In accord with the proposed structure, vibrations associated with a coordinated cysteine, two *cis*-oxo groups and the dithiolene of a single MPT are identified. This is the first time that Mo=O stretching modes have been observed for a biological molybdenum center, and in agreement with a direct oxo-transfer catalytic mechanism, only one is exchangeable with H_2O during redox cycling.

The Mo domains of the wild-type and C207S mutant forms of hSO were prepared by tryptic cleavage of the K108R and K108R/C207S mutant forms of hSO as previously described.[7]

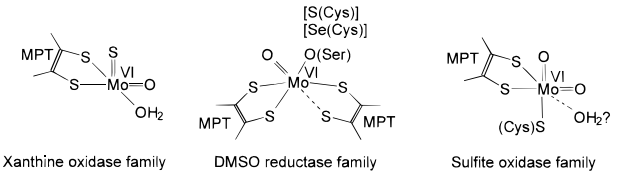

Figure 1. Active site structures for oxomolybdenum enzymes. The xanthine oxidase family structure is based on the X-ray crystal structure for *D. gigas* aldehyde oxidoreductase.[2] The DMSO reductase family structure is based on the X-ray crystal structure of *R. sphaeroides* DMSO reductase.[3] The proposed structure for the sulfite oxidase family is based on spectroscopic and mutagenesis data (see ref 4d).

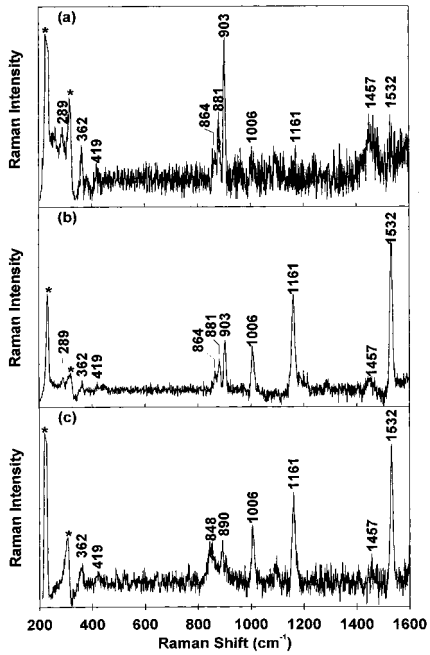

Figure 2. Resonance Raman spectra of the Mo domain (Mo(VI) oxidation state) of wild-type recombinant human sulfite oxidase: (a) as prepared, 514 nm excitation; (b) as prepared, 488 nm excitation; (c) after exchange into $H_2{}^{18}O$ buffer followed by dithionite reduction and reoxidation, 488 nm excitation. The samples (∼0.5 mM in Mo) were in 50 mM Tricine buffer, pH 8.0. The spectra were obtained by collecting scattered light from the surface of a frozen droplet at 25 K. Each spectrum is the sum of 50 scans with each scan involving photon counting for 1 s every 0.5 cm^{-1} with 6 cm^{-1} spectral resolution. Bands marked with an asterisk are lattice modes of the frozen buffer solution. A linear ramp has been subtracted to correct for the sloping fluorescence background.

Resonance Raman spectra using both 514-nm and 488-nm laser excitation, for the Mo(VI) forms of the wild-type and C207S samples, are shown in Figures 2 and 3, respectively, and the inset in Figure 3 shows the absorption spectra for the samples used in this work.[8] Identical RR spectra were observed for three distinct preparations and parallel studies with partially purified samples of the hSO heme domain showed that none of the observed bands arise from contaminating heme.[9] The spectra of the native protein were unchanged by redox cycling or by exchange into the equivalent $H_2{}^{18}O$ buffer using centricon ultrafiltration (except for the lattice modes of ice). However,

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(1) For a recent review, see: Hille, R. *Chem. Rev.* **1996**, *96*, 2757.
(2) (a) Romão, M. J.; Archer, M.; Moura, I.; Moura, J. J. G.; LeGall, J.; Engh, R.; Schneider, M.; Hof, P.; Huber, R. *Science* **1995**, *270*, 1170. (b) Huber, R.; Hof, P.; Duarte, R. O.; Moura, J. J. G.; Moura, I.; Liu, M.-Y.; LeGall, J.; Hille, R.; Archer, M.; Romão, M. J. *Proc. Natl. Acad. Sci U.S.A.* **1996**, *93*, 8846.
(3) (a) Schindelin, H.; Kisker, C.; Hilton, J.; Rajagopalan, K. V.; Rees, D. C. *Science* **1996**, *272*, 1615. (b) Schneider, F.; Löwe, J.; Huber, R.; Schindelin, H.; Kisker, C.; Knäblein, J. *J. Mol. Biol.* **1996**, *263*, 53.
(4) (a) Cramer, S. P.; Gray, H. G.; Rajagopalan, K. V. *J. Am. Chem. Soc.* **1979**, *101*, 2772. (b) Cramer, S. P.; Wahl, R.; Rajagopalan, K. V. *J. Am. Chem. Soc.* **1981**, *103*, 7721. (c) George, G. N.; Kipke, C. A.; Prince, R. C.; Sunde, R. A.; Enemark, J. H.; Cramer, S. P. *Biochemistry* **1989**, *28*, 5075. (d) George, G. N.; Garrett, R. M.; Prince, R. C.; Rajagopalan, K. V. *J. Am. Chem. Soc.* **1996**, *118*, 8588.
(5) (a) Lamy, M. T.; Gutteridge, S.; Bray, R. C. *Biochem. J.* **1980**, *185*, 397. (b) Bray, R. C.; Gutteridge, S.; Lamy, M. T.; Wilkinson, T. *Biochem. J.* **1983**, *211*, 227. (c) Dhawan, I. K.; Enemark, J. H. *Inorg. Chem.* **1996**, *35*, 4873.
(6) Garrett, R. M.; Rajagopalan, K. V. *J. Biol. Chem.* **1994**, *269*, 272.
(7) Garrett, R. M.; Rajagopalan, K. V. *J. Biol. Chem.* **1996**, *271*, 7387.

(8) Raman spectra were recorded with an Instruments SA U1000 scanning spectrometer fitted with a cooled RCA 31034 photomultiplier tube, using lines from a Coherent Innova 100 10-W Ar^+ or 200-K2 Kr^+ laser. Scattering was collected at 90° from the surface of a frozen droplet of protein solution on the cold finger of an Air Products Displex model CSA-202E closed cycle refrigerator (∼25 K). The fluorescence background was minimized by laser exposure for 5 h prior to data collection.
(9) A minor trace of the heme domain was apparent in resonance Raman data obtained with 406 nm excitation.

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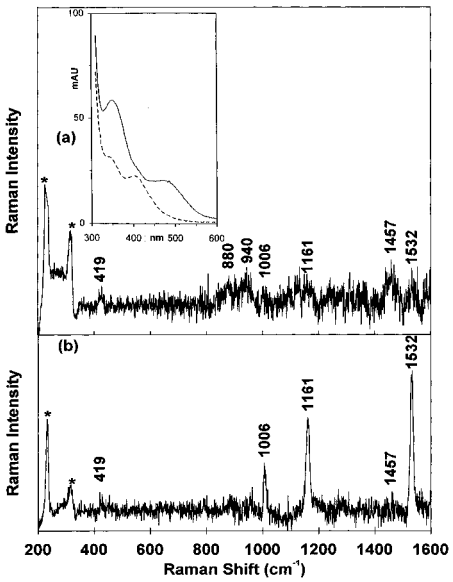

Figure 3. Resonance Raman spectra of the molybdenum domain (Mo(VI) oxidation state) of the C207S mutant of recombinant human sulfite oxidase: (a) as prepared, 514 nm excitation, (b) as prepared, 488 nm excitation. All other conditions and data handling protocols are as described in the legend to Figure 2. The inset shows a comparison of the absorption spectra of the wild-type (solid line) and C207S mutant (dashed line) samples used in this work.

the bands at 903 and 881 cm^{-1} are replaced by bands at 890 and 848 cm^{-1} in samples that were exchanged into $H_2{}^{18}O$ buffer and then redox cycled by dithionite reduction and air oxidation. This observation identifies both as Mo═O stretching modes, and by analogy with the available vibrational data for *cis*-dioxomolybdenum(VI) thiolate complexes ($\nu_{sym}(MoO_2) = 858-938$ cm^{-1}, 20−40 cm^{-1} higher in energy and stronger in the Raman spectrum than $\nu_{asym}(MoO_2) = 835-898$ cm^{-1}),[10] the 903- and 881-cm^{-1} bands in Figure 2a are clearly assignable to the symmetric and asymmetric stretches of a *cis*-MoO_2 unit. Solely on the basis of the increase in mass, these bands would be predicted to shift to 859 and 838 cm^{-1}, respectively, if both oxo groups were replaced by ^{18}O. Since the total $^{16}O/^{18}O$ isotope shift observed for these bands is almost exactly half of that predicted for exchange of both oxo groups, we conclude that only one oxo group exchanges during redox cycling. This result and the observed inequivalence in the isotope shifts for the symmetric and asymmetric stretching modes are predicted by normal mode calculations for dioxomolybdenum(VI) complexes.[11]

Resonant enhancement of the Mo═O stretching modes in wild-type hSO molybdenum domain was observed with excitation frequencies within the envelope of the absorption centered at 480 nm (i.e., strong enhancement with 514-, 488-, 476-, and 457-nm excitation and negligible enhancement with 406-, 568-, and 647-nm excitation). This electronic transition has been attributed to cysteinyl S-to-Mo(VI) charge transfer,[7] since it is lost in the C207S mutant (see inset in Figure 3). In accord with this assignment, the bands at 289 and 362 cm^{-1} that are observed with 514-, 488-, 476-, and 457-nm excitation of the wild-type, but are not present in the C207S mutant, are assigned to the $S_\gamma-C_\beta-C_\alpha$ bending and Mo−S stretching modes, respectively, of a coordinated cysteine residue. On the basis of model compound studies,[10f] this resonant enhancement behavior suggests that the cysteinyl S is likely to be *trans* to one of the oxo ligands, as depicted in Figure 1.

Recent X-ray absorption studies concluded that the C207S mutant of hSO contains a novel trioxomolybdenum species with two thiolate ligands from the dithiolene of MPT.[4d] Resonance Raman spectra of the molybdenum domain of the C207S mutant have been obtained with 406-, 457-, 488-, and 514-nm excitation, but the data are inconclusive concerning the presence of a MoO_3 species. Weak, broad bands that are potential candidates for Mo═O stretching are observed at 880 and 940 cm^{-1} (see Figure 3). However, the bands are too weak for meaningful isotope substitution experiments, presumably due to the loss of cysteinyl S-to-Mo(VI) charge transfer transition that appears to provide the enhancement mechanism for the Mo═O stretching modes in the wild-type.

Both the wild-type and C207S mutant of the molybdenum domain exhibit bands at 1006, 1161, and 1532 cm^{-1} that are not significantly enhanced with excitation wavelengths ≥514 nm, but are dominant bands in the spectra obtained with 488-, 476-, and 457-nm excitation (Figures 2 and 3). By analogy with vibrational spectra and normal mode calculations of metal dithiolene complexes,[12] these bands are readily assigned to vibrations associated with the coordinated dithiolene of MPT. The 1006 and 1161 cm^{-1} bands are assigned to coupled C−S and C−C stretching modes (C−S and C−C stretching as the dominant contributors at 1006 and 1161 cm^{-1}, respectively) and the 1532-cm^{-1} band is assigned to C═C stretching.[13] Using excitation at wavelengths >600 nm into low-energy S → Mo^{VI} charge transfer bands, both dithiolene Mo−S and C═C stretching modes have been observed in the resonance Raman spectra of *R. sphaeroides* DMSO reductase (ν_{sym}(Mo−S) = 350 cm^{-1} and ν(C═C) = 1526 cm^{-1} and/or 1576 cm^{-1} in the Mo(VI) oxidation state)[14] and in dioxomolybdenum(VI) complexes with two dithiolene ligands (ν_{sym}(Mo−S) = 354 cm^{-1} and ν(C═C) = 1455−1503 cm^{-1}).[10e] The weak band at 419 cm^{-1} that is observed in both wild-type and C207S hSO is a potential candidate for a dithiolene Mo−S stretching mode. However, it is clear that such vibrational modes are only weakly enhanced in hSO, and this may be a consequence of greater π-dithiolene-to-Mo charge transfer character (as opposed to S-to-Mo character) in the higher energy electronic transitions that are associated with Mo centers coordinated by a single dithiolene ligand. This observation has important consequences for the emerging understanding of both the electronic and vibrational properties of the Mo and W active sites of oxotransferases. For example, it provides a rationalization for the dramatic differences in the energy range and variable-temperature MCD intensity of transitions involving biological W(V)/Mo(V) centers with one or two dithiolene ligands.[15]

Acknowledgment. This work was supported by grants from the NSF (MCB9419019 to M.K.J.) and the NIH (GM00091 to K.V.R.).

JA963931C

(10) (a) Ueyama, N.; Nakata, M.; Araki, T.; Nakamura, A.; Yamashita, S.; Yamashita, T. *Inorg. Chem.* **1981**, *20*, 1934. (b) Kaul, B. B.; Enemark, J. H.; Merbs, S, L.; Spence, J. T. *J. Am. Chem. Soc.* **1985**, *107*, 2885. (c) Willis, L. J.; Loehr, T. M.; Miller, K. F.; Bruce, A. E.; Stiefel, E. I. *Inorg. Chem.* **1986**, *25*, 4289. (d) Subramanian, P.; Burgmayer, S.; Richards, S.; Szalai, V.; Spiro, T. G. *Inorg. Chem.* **1990**, *29*, 3849. (e) Oku, H.; Ueyama, N.; Nakamura, A. *Inorg. Chem.* **1995**, *34*, 3667. (f) Ueyama, N.; Oku, H.; Kondo, M.; Okamura, T.; Yoshinaga, N.; Nakamura, A. *Inorg. Chem.* **1996**, *35*, 643.

(11) Willis, L. J.; Loehr, T. M. *Spectrochim. Acta* **1987**, *43A*, 51.

(12) (a) Schläpfer, C. W.; Nakamoto, K. *Inorg. Chem.* **1975**, *14*, 1338. (b) Clark, R. J. H.; Turtle, P. C. *J. Chem. Soc., Dalton Trans.* **1978**, 1714.

(13) The only other dithiolene mode that might be expected in the high-frequency region would be a C−S stretch near 850 cm^{-1},[12] and the weak band at 864 cm^{-1} in the wild-type is clearly a good candidate for this mode. Alternatively, the 864 cm^{-1} band could be interpreted as an additional Mo═O mode, raising the possibility of previously undetected active site heterogeneity in the Mo(VI) oxidation state. RR studies of the molybdenum domain of hSO as a function of pH and anion concentration are planned to address this issue. The origin of the band at 1457 cm^{-1} band that is observed for wild-type and the C207S mutant is unclear at present.

(14) (a) Gruber, S.; Kilpatrick, L.; Bastian, N. R.; Rajagopalan, K. V.; Spiro, T. G. *J. Am. Chem. Soc.* **1990**, *112*, 8179−8180. (b) Kilpatrick, L.; Rajagopalan, K. V.; Hilton, J.; Bastian, N. R.; Stiefel, E. I.; Pilato, R. S.; Spiro, T. G. *Biochemistry* **1995**, *34*, 3032−3039.

(15) Koehler, B. P.; Mukund, S.; Conover, R. C.; Dhawan, I. K.; Roy, R.; Adams, M. W. W.; Johnson, M. K. *J. Am. Chem. Soc.* **1996**, *118*, 12391−12405 and references cited therein.