Resonance Raman Characterization of Biotin Sulfoxide Reductase
COMPARING OXOMOLYBDENUM ENZYMES IN THE Me₂SO REDUCTASE FAMILY*

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Resonance Raman spectroscopy has been used to define active site structures for oxidized Mo(VI) and reduced Mo(IV) forms of recombinant Rhodobacter sphaeroides biotin sulfoxide reductase expressed in Escherichia coli. On the basis of ¹⁸O/¹⁶O labeling studies involving water and the alternative substrate dimethyl sulfoxide and the close correspondence to the resonance Raman spectra previously reported for dimethyl sulfoxide reductase (Garton, S. D., Hilton, J., Oku, H., Crouse, B. R., Rajagopalan, K. V., and Johnson, M. K. (1997) J. Am. Chem. Soc. 119, 12906–12916), vibrational modes associated with a terminal oxo ligand and the two molybdopterin dithiolene ligands have been assigned. The results indicate that the enzyme cycles between mono-oxo-Mo(VI) and des-oxo-Mo(IV) forms with both molybdopterin dithiolene ligands remaining coordinated in both redox states. Direct evidence for an oxygen atom transfer mechanism is provided by ¹⁸O/¹⁶O labeling studies, which show that the terminal oxo group at the molybdenum center is exchangeable with water during redox cycling and originates from the substrate in substrate-oxidized samples. Biotin sulfoxide reductase is not reduced by biotin or the nonphysiological products, dimethyl sulfide and trimethylamine. However, product-induced changes in the Mo=O stretching frequency provide direct evidence for a product-associated mono-oxo-Mo(VI) catalytic intermediate. The results indicate that biotin sulfoxide reductase is thermodynamically tuned to catalyze the reductase reaction, and a detailed catalytic mechanism is proposed.

With the exception of nitrogenase, molybdenum enzymes catalyze formal oxygen atom transfer between water and substrate and contain an active site in which the molybdenum is coordinated by the dithiolene side chain of one or two molybdopterins (Fig. 1a) (1–3). Although the recent proliferation of x-ray crystal structures for mononuclear molybdenum enzymes has revealed a common structure for the molybdopterin cofactor, there have also revealed considerable diversity in the molybdenum coordination environment (2–11). However, on the basis of the available crystallographic, spectroscopic, primary sequence, and cyanide inhibition data, these enzymes can be divided into three large families (Fig. 1b) (1). Hydroxylases, such as Desulfovibrio gigas aldehyde oxidoreductase, xanthine oxidase, and xanthine dehydrogenase, represent the xanthine oxidase family, characterized by a Mo(VI) active site with a single molybdopterin, as well as terminal oxo and sulfido groups. The oxotransferase class of molybdenoenzymes catalyzes direct oxygen atom transfer between substrate and water and can be divided into two families. The sulfite oxidase family, as represented by sulfite oxidase and assimilatory nitrate reductase, contains Mo(VI) ligated by two oxo groups in addition to a single molybdopterin and a cysteine residue. The dimethyl sulfoxide (Me₂SO) reductase family comprises mono-oxo-Mo(VI) sites ligated by two molybdopterins and a protein side chain ligand such as serine, cysteine, or selenocysteine. In addition to Me₂SO reductase, this family also includes formate dehydrogenase, dissimilatory nitrate reductase, and biotin sulfoxide (BSO)1 reductase.

BSO reductase is found in bacterial systems, such as Rhodobacter sphaeroides and Escherichia coli, and catalyzes the reduction of d-biotin d-sulfoxide to d-biotin (Fig. 2). Although the precise role for BSO reductase in bacterial metabolism has yet to be defined, potential physiological functions include scavenging biotin sulfoxide from the environment, reducing bound intracellular biotin that has become oxidized in an aerobic environment, and protecting the cell from oxidative damage (12). Extensive characterization of this enzyme has been limited due to the low natural abundance of the protein and its constitutive expression (13). However, R. sphaeroides BSO reductase has recently been heterologously expressed in E. coli and characterized as containing the molybdopterin guanine dinucleotide form of the molybdopterin cofactor (14). Using either reduced methyl viologen or NADPH as electron donor, the enzyme was found to exhibit broad substrate specificity and was able to catalyze reduction of nicotinamide-N-oxide, methionine sulfoxide, trimethylamine-N-oxide, and dimethyl sulfoxide with varying efficiencies, in addition to the reduction of biotin sulfoxide (14). Recent improvements in the expression system have produced higher yields of recombinant BSO reductase (15), making the enzyme more readily available for extensive spectroscopic characterization.

BSO reductase from R. sphaeroides and E. coli and Me₂SO reductase from R. sphaeroides and Rhodobacter capsulatus are members of a small class of molybdenum oxotransferases that contain the mononuclear molybdenum center as their sole prosthetic group, making them highly amenable to spectroscopic investigations to elucidate the electronic and vibrational properties

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1 The abbreviations used are: BSO, biotin sulfoxide; EXAFS, extended X-ray absorption fine structure; TMA, trimethylamine.

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different from the spectroscopically deduced structures for molybdopterin dithiolanes. Moreover, the three structures are number of terminal oxo groups and/or the attachment of the and reduced forms, but each differs from the others in terms of (4–6), concur in finding a serinate ligand in both the oxidized forms, but with a typical terminal Mo=O group (1.7 Å) due to hydrogen bonding to a tryptophan residue, and it is this oxo group that constitutes the site of attack by Me_S (5, 6, 20). However, a catalytic scheme involving a spectator oxo group and a long terminal oxo group that is poised for oxygen atom transfer is not consistent with the resonance Raman and EXAFS data for R. sphaeroides Me_SO reductase (16, 19). In addition to the absence of any evidence for a spectator oxo group in dithionite-or Me_S-reduced samples, the vibrational frequency of the substrate- and solvent-exchangeable oxo group can only be interpreted in terms of a short Mo=O (i.e. ~1.7 Å).

The discrepancies between the spectroscopically and crystallographically defined structures of Me_SO reductase and the possibility that more than one structural form of the active site is catalytically competent provided the impetus for the current resonance Raman study of another member of the Me_SO reductase family, R. sphaeroides BSO reductase. Sequence comparisons of BSO reductase and Me_SO reductase indicate a high degree of homology (50% identity). In particular, the residues involved in interaction with the two molybdopterin guanine dinucleotide units and the serine that ligates Mo are all strictly conserved (13, 14). In this work, we report on resonance Raman studies of R. sphaeroides BSO reductase in oxidized and reduced forms in the presence and absence of substrates and products. As for Me_SO reductase (16), the results indicate a direct oxygen atom transfer mechanism involving mono-oxo-Mo(VI) and des-oxo-Mo(IV) species with the dithiolanes of both molybdopterin remaining coordinated throughout the catalytic cycle. In addition, the resonance Raman studies of BSO reductase have enabled characterization of the product-bound Mo(VI) intermediate that was proposed (16), but not observed, in resonance Raman studies of Me_SO reductase. The inability of biotin to reduce the Mo(VI) active site of BSO reductase indicates that the enzyme is thermodynamically tuned to catalyze the reductase reaction.

EXPERIMENTAL PROCEDURES

BSO Reductase Samples—Heterologous expression of R. sphaeroides BSO reductase in E. coli has been reported in the literature by Pollock and Barber (14) Resonance Raman samples were prepared using a modified procedure that results in a much greater yield of recombinant enzyme (15). Samples were prepared in 50 mM tricine buffer, pH 8.0, and were concentrated to ~4 mM for resonance Raman studies by Centricron ultrafiltration. Exchange into the equivalent 18O buffer (prepared using H_218O with 95–98% isotopic enrichment obtained from Cambridge Isotope Laboratories) was carried out by three 10-fold-dilution and reconcentration cycles. Dithionite reduction was carried out anaerobically under argon in a Vacuum Atmospheres glovebox (21±1 ppm O_2) by addition of a 20-fold excess of sodium dithionite from a stock solution in pH 8.0 buffer. Oxidation was accomplished by addition of BSO (freshly prepared according to the method of Pollack and Barber (13)), Me_SO, or ferricyanide. Excess ferricyanide was removed from the sample by three dilution/reconcentration cycles. 18O-Enriched Me_SO (95% Me_S18O) was prepared as described previously (16). Biotin, Me_S, and trimethylamine (TMA) were purchased from Aldrich and were added to the oxidized samples in a 10-fold stoichiometric excess.

Spectroscopic Methods—UV-visible absorption spectra were recorded in 1-mm cuvettes using a Shimadzu UV301PC spectrophotometer. Resonance Raman spectra were recorded using an Instruments SA Raman U1000 spectrometer fitted with a cooled RCA 31034 photomultiplier tube with 90° scattering geometry. Spectra were recorded digitally using photon counting electronics, and improvements in signal-to-noise were achieved by signal averaging multiple scans. Band positions were calibrated using the laser excitation frequency and CCl_4.
and are accurate to ±1 cm⁻¹. Lines from a Coherent Innova 200-K2 krypton ion laser or Coherent Innova Sabre 10-W argon ion laser were used for excitation, and plasma lines were removed using a Pellin Broca Prism premonochromator. For each sample, 70–100 mW of incident laser power was used, and slit widths were adjusted for each excitation wavelength to give 6.5 cm⁻¹ spectral resolution. Scattering was collected from the surface of a frozen 10-µl droplet of sample using a custom-designed anaerobic sample cell (21) attached to the cold finger of an Air Products Diaplex model CSA-202E closed cycle refrigerator. This arrangement enables samples to be cooled down to 18 K, which facilitates improved spectral resolution and prevents laser-induced sample degradation. For each sample, the first scan exhibited the same Raman bands as the final scan, verifying lack of sample degradation or redox processes. Moreover, the room temperature UV-visible absorption spectra of all samples were identical before and after Raman data accumulation. The only difference between first and last scan during Raman data acquisition resulted from the progressive decrease in the fluorescence background with continuous laser exposure at 18 K. A linear ramp fluorescent background has been subtracted from each spectrum shown in this work.

RESULTS

The UV-visible absorption spectra of as prepared and dithionite-reduced recombinant R. sphaeroides Me₅SO reductase are very similar to those of the corresponding samples of wild-type R. sphaeroides Me₅SO reductase (15). As prepared, BSO reductase has maxima at 350 (ε = 5.3 mm⁻¹ cm⁻¹), 570 (ε = 2.2 mm⁻¹ cm⁻¹), and 720 nm (ε = 1.8 mm⁻¹ cm⁻¹) and a shoulder at ~470 nm (ε = 2.6 mm⁻¹ cm⁻¹) (15), compared with maxima at 350 (ε = 4.8 mm⁻¹ cm⁻¹), 550 (ε = 1.8 mm⁻¹ cm⁻¹), and 720 nm (ε = 2.0 mm⁻¹ cm⁻¹) and a shoulder at ~470 nm (ε = 2.3 mm⁻¹ cm⁻¹) for as-prepared wild-type R. sphaeroides Me₅SO reductase (22). The low energy transition in the near IR region is characteristic of the bacterial Me₅SO reductase family in which the Mo center is coordinated by the dithiolenes of bis(molybdopterin guanine dinucleotide)moi- lybdopterins. In the case of Me₅SO reductase, resonance Raman studies indicated that the absorption features above 500 nm arise from thiolate-to-Mo(VI) charge transfer transitions, as concluded for Me₅SO reductase (12). The Mo–S stretching region of as-isolated Mo(VI) BSO reductase (24, 25) for Me₅SO reductase, because no isotope shift data is available for BSO reductase. For excitation wavelengths in the range 457–647 nm, the dominant feature in the Mo–S stretching region of as-prepared Mo(VI) BSO reductase is the totally symmetric breathing mode of the MoS₄ unit at 355 cm⁻¹ (23). The analysis of the MoS₄ unit in Me₅SO reductase utilized [MoOCl₄]²⁻ as a frame of reference (16), and the same overall pattern is expected for BSO reductase, albeit at shifted frequencies. Therefore, the criteria used for assigning Mo–S vibrational modes for BSO reductase (Table I) include the following: similarity to both Me₅SO reductase and relevant model complexes such as [MoOCl₄]²⁻, sensitivity to Mo oxidation state, and the ³⁴S isotope shift data published by Spiro and co-workers (24, 25) for Me₅SO reductase, because no isotope shift data is available for BSO reductase. For excitation wavelengths in the range 457–647 nm, the dominant feature in the Mo–S stretching region of as-prepared Mo(VI) BSO reductase is the totally symmetric breathing mode of the MoS₄ unit at 355 cm⁻¹. This stretching mode, ν₁(A₁)(MoS₄), correlates to the feature observed at 350 cm⁻¹ for Me₅SO reductase (Table I). The strong enhancement of the A₁ mode throughout the visible and near IR region indicates that the absorption bands in these regions arise from dithiolate-to-Mo(VI) charge transitions, as concluded for Me₅SO reductase (12). The ν₁(A₁)(MoS₄) mode is flanked by the lower energy ν₅(B₁)(MoS₄) mode at 331 cm⁻¹, and the two components of the higher energy ν₄(E)(MoS₄) mode are observed at 368 and 378 cm⁻¹, split by lower symmetry imposed by the dithiolene chelates and the non-sulfur ligands. Similar features are observed for Me₅SO reductase, i.e. a ν₅(B₁)(MoS₄) mode at 336 cm⁻¹ and the higher energy ν₅(E)(MoS₄) modes at 370 and 377 cm⁻¹ (Table I). In the case of Me₅SO reductase, these assignments are supported by ³⁴S isotope shifts (16, 24, 25). The average Mo–S stretching frequency is unchanged compared with Me₅SO reductase indicating similar Mo–S(dithiolene) bond strengths in these two enzymes. Split modes for the components of the deformation mode, π₆(E)(MoS₄), were tentatively assigned at 250 and 260 cm⁻¹ for the Mo(VI) form of Me₅SO reductase (16), and by
analogy, the features at 242 (shoulder on 231 cm\(^{-1}\) ice peak) and 268 cm\(^{-1}\) are tentatively assigned to the same modes in BSO reductase.

Dithionite reduction of BSO reductase produces a dramatic change in the resonance Raman spectrum in the Mo–S stretching region, but the intensity pattern and frequencies are similar to those of dithionite-reduced Me\(\text{2SO}\) reductase and can be assigned by direct analogy (Table I). Therefore, the 363-cm\(^{-1}\) mode of BSO reductase is assigned as \(\nu_1(B_2\text{(MoS}_4\text{)})\), with the 386-cm\(^{-1}\) and 405-cm\(^{-1}\) modes assigned as components of \(\nu_1(E\text{MoS}_4\text{)}\). A weak band at 345-cm\(^{-1}\) that is only clearly apparent with 530-nm excitation (data not shown) is assigned to the \(\nu_1(B_1\text{(MoS}_4\text{)})\) mode. In Me\(\text{2SO}\) reductase, equivalent modes have been assigned at 367, 385, 402, and 346 cm\(^{-1}\), respectively, on the basis of \(^{34}\text{S}\) isotope shifts (16). On reduction from Mo\(\text{VI}\) to Mo\(\text{IV}\) for BSO reductase, the increase in average Mo–S frequencies with decreasing oxidation state indicates stronger Mo–S bonds in the reduced state. This trend on reduction was also observed in resonance Raman and EXAFS studies of Me\(\text{2SO}\) reductase (16, 18, 19) and is attributed to strengthening of the Mo–S bonds in response to loss of the oxo ligand.

The bands at 288, 416, and 447 cm\(^{-1}\) do not change significantly as a function of the Mo oxidation state and are also observed in Me\(\text{2SO}\) reductase. Because the frequencies of these bands are invariant to Mo oxidation state in both enzymes and impervious to \(^{34}\text{S}\) isotopic substitution of the dithiolate sulfurs in Me\(\text{2SO}\) reductase (16, 24, 25), these modes are attributed to dithiolene- or pyran-ring deformations. This leaves the bands at 543 and 436 cm\(^{-1}\) in as-prepared BSO reductase and at 495 and 425 cm\(^{-1}\) in dithionite-reduced BSO reductase as potential candidates for Mo–O(Ser) stretching. The possibility that one of these modes in the reduced sample arises from Mo–OH\(_2\) stretching was ruled out by the absence of any significant isotope shift for samples exchanged into H\(_{18}\text{O}\) buffer (data not shown). In Me\(\text{2SO}\) reductase, Mo–O(Ser) stretching modes were tentatively assigned to bands at 536 and 513 cm\(^{-1}\) in the Mo\(\text{VI}\) and Mo\(\text{IV}\) forms, respectively (12). This assignment has recently been supported by the loss of these bands upon mutation of Ser-147 to a cysteine residue in Me\(\text{2SO}\) reductase (26).

In the absence of mutagenesis or isotopic labeling studies, more definitive assignment of the Mo–O(Ser) stretching modes in BSO reductase is not possible.

Mo–O Stretching Modes—As-prepared recombinant BSO reductase samples have an intense broad band centered at 860 cm\(^{-1}\) that dominates the mid-frequency region of the resonance Raman spectrum using visible excitation in the range 488–568 nm. A similar dominant band, centered at 862 cm\(^{-1}\), although not quite as broad, was identified as a Mo–O stretching mode in as-prepared wild-type Me\(\text{2SO}\) reductase via isotopic labeling studies (16). As in Me\(\text{2SO}\) reductase, the 860-cm\(^{-1}\) band in BSO reductase is completely lost on dithionite reduction (Fig. 4), suggesting a des-oxo-Mo\(\text{IV}\) formulation for the reduced enzyme. The reduced sample has a broad band centered at 760 cm\(^{-1}\), but this corresponds to the first overtone of the intense Mo–S stretching modes. In the as prepared sample, the first overtone of the Mo–S stretching modes is seen as a broad band centered at 710 cm\(^{-1}\). Hence, the band at 860 cm\(^{-1}\) in as prepared BSO reductase is a good candidate for the Mo–O stretching mode of a mono-oxo-Mo\(\text{VI}\) unit, with the breadth reflecting heterogeneity in the active site structure or environment.

Two types of isotope labeling experiments were carried out with BSO reductase to test this hypothesis and assess whether this putative terminal oxo is exchangeable with solvent and substrates.

Fig. 5 shows the resonance Raman spectra that result from dithionite reduction of BSO reductase followed by anaerobic buffer exchange using H\(_{18}\text{O}\) and H\(_{17}\text{O}\) tricine buffers and reoxidation with ferricyanide. Redox cycling in H\(_2\)\text{18O} buffer resulted a spectrum almost identical to that of the as-prepared recombinant enzyme. Other than the anticipated shifts of the lattice modes of ice, the only significant change in the sample reoxidized in H\(_2\)\text{18O} buffer is the shift of the 860-cm\(^{-1}\) band to 818 cm\(^{-1}\). This confirms the assignment of this band as a Mo–O stretching mode involving a solvent-exchangeable oxo group. Moreover, the \(^{18}\text{O}/^{16}\text{O}\) isotope shift, 42 cm\(^{-1}\), is exactly that predicted for a single Mo–O bond based on the mass effect using a simple diatomic oscillator approximation, indicating a mono-oxo-Mo\(\text{VI}\) site. Two bands, each with a much smaller isotope shift, are expected and observed for di-oxo-Mo centers in which only one of the oxo groups is exchangeable (17, 27, 28).

The ability of BSO to reoxidize dithionite-reduced BSO reductase has been demonstrated by both UV-visible absorption and resonance Raman studies. The resonance Raman spectrum of a BSO reoxidized BSO reductase (Fig. 6a) is very similar to that of the as-prepared and redox cycled sample, except for narrower and better resolved bands associated with dithiolene ring stretching (see below) and Mo–O stretching. Two bands are resolved in the Mo–O stretching region: one at 860 cm\(^{-1}\), the same frequency as observed in the as-prepared and redox cycled samples, and the other, a weaker band, at 890 cm\(^{-1}\). Unfortunately, it has not been possible to confirm assignment of these bands as Mo–O stretching modes and demonstrate direct oxygen atom transfer between BSO and BSO reductase.
because our attempts to make $^{18}$O-labeled BSO starting with biotin have thus far proven unsuccessful. Consequently resonance experiments with $^{18}$O-labeled substrate were carried out with an alternative substrate, Me$_2$SO (14).

When BSO reductase is reduced with dithionite and then reoxidized with Me$_2$S$^{18}$O rather than ferricyanide, the Mo=O stretching region comprises an intense and much sharper band at 840 cm$^{-1}$ and a weaker band at 860 cm$^{-1}$ (Fig. 6b). Other than these changes in the Mo=O stretching mode, the spectrum is very similar to that of as-prepared BSO reductase, suggesting that active site heterogeneity resides in interactions involving the terminal oxo group. Reduction with dithionite and subsequent reoxidation in H$_2^{16}$O buffer with labeled Me$_2$S$^{15}$O resulted in two major bands at Mo=O stretching bands at 797 and 840 cm$^{-1}$ (data not shown). The spectrum is indicative of a Mo(VI) site, and excess substrate was evident in the Raman spectrum, in accord with oxidation of the sample. This indicates a mixture of Mo=$^{16}$O and Mo=$^{15}$O sites, because a 43-cm$^{-1}$$^{18}$O downshift would be expected for a monooxo-Mo(VI) site with a Mo=$^{16}$O stretching frequency at 840 cm$^{-1}$. Hence, this result suggests that the terminal oxo group in Me$_2$SO-oxidized BSO reductase is labile to solvent exchange without redox cycling. In contrast, no significant exchange of the oxo group was apparent in samples of asprepared BSO reductase that were exchanged into a H$_2^{18}$O buffer and left in a refrigerator at 4 °C for 4 h, as evidenced by resonance Raman studies (data not shown).

Two experiments were undertaken to assess whether the terminal oxo group in Me$_2$SO-reoxidized BSO reductase is solvent-exchangeable. In the first experiment, the BSO reductase was reduced with dithionite in H$_2^{16}$O buffer, reoxidized with Me$_2$S$^{15}$O (Fig. 6a). Without the availability of H$_2^{18}$O in the sample, the resulting Mo=O stretching region comprised an intense band at 797 cm$^{-1}$ and a weaker band at 860 cm$^{-1}$, i.e. a spectrum identical to that obtained for the sample turned over with unlabeled Me$_2$SO. In the second experiment, BSO reductase was reduced with dithionite after exchange into H$_2^{18}$O tricine buffer and reoxidized with Me$_2$S$^{18}$O. Without the availability of H$_2^{16}$O in the sample, the resulting Mo=O stretching region comprises an intense band at 797 cm$^{-1}$ and a weaker band at 818 cm$^{-1}$ (Fig. 6c). The insensitivity of the bands in the Mo=O stretching region to the mass of the terminal oxo ligand, coupled with the fully developed $^{18}$O/$^{16}$O isotope shift, demonstrates that the Mo=O stretching is not significantly coupled with any other vibration. Because both of the two Mo=O stretching modes exhibit 42–43 cm$^{-1}$ $^{18}$O downshifts, we conclude that there are two mono-oxo-Mo(VI) species present in Me$_2$SO-reoxidized samples and, by analogy, in the BSO-reoxidized samples discussed above. The species with $\nu$(Mo=O) = 860 cm$^{-1}$ is common to both Me$_2$SO- and BSO-reoxidized samples, as well as the as-prepared and redox cycled enzyme, and the other has $\nu$(Mo=O) = 840 cm$^{-1}$ in Me$_2$SO-reoxidized samples and $\nu$(Mo=O) = 890 cm$^{-1}$ in BSO-reoxidized samples. As shown below, these two new forms correspond to product bound forms of the oxidized enzyme. Overall, these experiments show that the oxo group on the substrate is transferred directly to the des-oxo-Mo(IV) active site to give a mono-oxo-Mo(VI) species that after oxidation with Me$_2$SO, the terminal oxo group is weaker and labilized toward solvent exchange.

Me$_2$SO reductase can be reduced by Me$_2$S, the product of the enzymatic reaction, to give a stable Me$_2$SO-bound Mo(IV) species that has been attributed to an intermediate in the catalytic cycle (6, 16, 19, 20). Direct evidence for the formation of a Me$_2$SO-bound Mo(IV) species with the oxo group of the mono-oxo-Mo(VI) active site providing the site of attack by Me$_2$S was provided by resonance Raman studies using enzyme in which the oxo group was isotopically labeled with $^{16}$O or $^{18}$O (16). In contrast, both UV-visible absorption and resonance Raman studies indicate that neither the physiological product, biotin, nor the nonphysiological products, Me$_2$S and TMA, were capable of reducing the Mo center in BSO reductase. The resonance Raman spectra that result from incubating as-prepared BSO reductase with a 20-fold stoichiometric excess of biotin, Me$_2$S.
and TMA are compared in Fig. 7. The addition of biotin did not significantly perturb the bands in the Mo–S stretching region but did result in an overall sharpening of all bands and a resolvable splitting in the Mo=O stretching band: a major component at 860 cm\(^{-1}\), analogous to that observed in the as-prepared enzyme, and a minor component at 890 cm\(^{-1}\). The resulting spectrum is almost identical to that of BSO reoxidized samples, indicating that the minor component at 890 cm\(^{-1}\) in this sample (Fig. 7a) corresponds to a biotin-bound form of the oxidized enzyme. Likewise, the Me\(\text{S}_2\)S-treated sample closely corresponds to the Me\(\text{S}_2\)-reoxidized sample, with the dominant Mo=O stretching mode at 840 cm\(^{-1}\). Hence, we conclude that the variation in the Mo=O stretching frequencies in BSO reductase arises from interaction between the oxo group and the product of the enzymatic reaction, with biotin exhibiting a weaker interaction than Me\(\text{S}_2\). The interaction of the oxo group with TMA is intermediate between that observed for Me\(\text{S}_2\) and biotin as judged by the Mo–O stretching frequency, 850 cm\(^{-1}\). However, the TMA-treated sample differs from the Me\(\text{S}_2\)- and biotin-treated samples in that there are significant changes in the Mo–S stretching region, namely a 5-cm\(^{-1}\) downshift in the \(\nu_2\)(A\(_1\)/Mo=O) stretching frequency to 350 cm\(^{-1}\) and stronger enhancement of the \(\nu_2\)(B\(_1\)/Mo=O) band at 331 cm\(^{-1}\) and one of the components of \(\nu_2\)(E/MoS\(_2\)) at 378 cm\(^{-1}\) (Fig. 7). Although the origin of these changes in the Mo–S stretching region of the resonance Raman spectrum are unclear at present, the close similarity in the Mo–S stretching frequencies compared with those of the as-prepared enzyme and the presence of a Mo=O stretching mode at 850 cm\(^{-1}\) and parallel UV-visible absorption studies argue against significant reduction by TMA.

**Dithiolene Ring Stretching Modes**—The molybdopterin dithiolene units of BSO reductase can each be viewed as a cis-C\(_5\)C\(_2\)C\(_5\)S\(_2\) unit under local C\(_2\)v symmetry (16, 29, 30). Estimates of the anticipated frequency ranges for each of the five stretching modes can be made on the basis of the detailed vibrational analysis of transition metal dithiolene complexes containing the equivalent unit, e.g. [Ni(S\(_2\)C\(_5\)CN\(_2\))\(_2\)]\(^{2-}\) (29) and [M(S\(_2\)C\(_5\)CN\(_2\))\(_2\)]\(^{2-}\) (M = V, Mo, W) (30): \(\nu(C=C)\), 760–900 cm\(^{-1}\); \(\nu(C–C)\), \(\nu(C–S)\) + \(\nu(C–S)\), and \(\nu(C–S) + \nu(C=C)\) all in the region 1000–1180 cm\(^{-1}\); \(\nu(C=C)\) in the range 1400–1600 cm\(^{-1}\).

Within these ranges, the \(\nu(C–S)\) and \(\nu(C=C)\) frequencies are inversely correlated with low \(\nu(C–S)\) and high \(\nu(C=C)\), indicating that the dithiolene is effectively acting as a dithiolate ligand, whereas high \(\nu(C–S)\) and low \(\nu(C=C)\) indicates substantial \(\pi\)-delocalization and some dithioketone character for the ligand. Several of the anticipated dithiolene ring stretching modes are observed in the high frequency region, in addition to nonresonantly enhanced Raman bands of the dithiolene ring stretching modes in dithionite-reduced Me\(\text{S}_2\)-reductase (Fig. 4). The dithiolene with the higher \(\nu(C=C)\) frequency, 1573 cm\(^{-1}\), is therefore best considered as a dithioketone ligand, and the \(\nu(C–S)\) mode is tentatively assigned to the weak band at 760 cm\(^{-1}\) (Fig. 4). The dithiolene with the lower \(\nu(C=C)\) frequency, 1529 cm\(^{-1}\), has a greater degree of \(\pi\)-delocalization and the corresponding \(\nu(C–S)\) mode probably lies under the broad Mo–O stretching mode in the 800–860 cm\(^{-1}\) region. The complicated set of weak bands at 1004, 1017, 1041, 1090, and 1128 cm\(^{-1}\) in as-prepared and at 1002, 1041, 1085, and 1124 cm\(^{-1}\) in dithiolene-reduced BSO reductase (Fig. 4) are attributed to \(\nu(C–C)\) stretching and combinations of \(\nu(C–C)\) and \(\nu(C–S)\) stretching of the dithiolene molybdopterins.

Only one type of dithiolene with \(\nu(C=C) = 1582\) cm\(^{-1}\) is clearly apparent for dithionite-reduced BSO reductase with 568-nm excitation, with each scan involving photon counting for 1 s every 1.0 cm\(^{-1}\) with 6.5 cm\(^{-1}\) resolution. The lattice modes of ice are denoted by an asterisk (*), and nonresonantly enhanced vibrational modes of the polypeptide backbone are indicated by P.
capsulatus (4–6) are in error due to mixtures of conformations in the crystalline samples or correspond to inactive conformations.

Direct evidence for different active site conformations of *R. sphaeroides* Me₂SO reductase in solution has recently come from Mo-EXAFS studies of recombinant *R. sphaeroides* Me₂SO reductase (19). As purified, the recombinant enzyme was shown to have a di-oxo-Mo(VI) structure, with both sets of molybdopterin dithiolenes coordinated. However, the enzyme is converted to the des-oxo-Mo(IV) form after reduction and yields the functional mono-oxo-Mo(VI) species upon reoxidation. This result and the effect of redox cycling have recently been confirmed by resonance Raman studies of recombinant *R. sphaeroides* Me₂SO reductase (26). The di-oxo-Mo(VI) species in the recombinant enzyme was found to have a distinctive resonance Raman spectrum, with two Mo=O stretching modes at 823 and 854 cm⁻¹ and the same pattern of Mo–S stretching modes, but shifted to lower frequencies (e.g. the intense ν₁(A₁)(MoS₄) symmetric stretching mode occurs at 332 cm⁻¹ in the recombinant enzymes compared with 350 cm⁻¹ in the wild-type enzyme). Hence, a di-oxo-Mo(VI) site in BSO reductase would be expected to be readily detectable by resonance Raman but has yet to be observed in BSO reductase.

The present study of BSO reductase demonstrates that resonance Raman provides a measure of the heterogeneity in the vicinity of the active site oxo group via the breadth and splitting of the Mo=O stretching mode. As prepared samples of recombinant BSO reductase exhibit broader Mo=O stretching modes than as-prepared samples of wild-type Me₂SO reductase, as judged by their bandwidth at half-height, 50 and 35 cm⁻¹, respectively. This suggests more conformational flexibility in the substrate/product binding pocket in BSO reductase, which is consistent with the larger size of the substrate, BSO. As in Me₂SO reductase, the Mo=O stretching band in BSO reductase does not narrow significantly on redox cycling in the absence of a substrate but does sharpen significantly on reoxidation with substrate, indicating that catalytic cycling reduces active site heterogeneity by locking in well-defined conformations. In the case of Me₂SO reductase, the bandwidth of the Mo=O stretching mode narrows to 20 cm⁻¹ on reoxidation with excess Me₂SO but is unchanged in frequency compared with the as-prepared enzyme, 862 cm⁻¹ (16). A similar sharpening occurs on reoxidation of BSO reductase with BSO and Me₂SO, but in both cases, the Mo=O stretching region comprises two resolved modes, each with a bandwidth of ~20 cm⁻¹. In light of the studies of the effects of biotin and Me₂S on the resonance Raman spectrum of BSO reductase, one of these Mo=O stretching modes is attributed to the product-bound form (840 cm⁻¹ for Me₂S and 890 cm⁻¹ for biotin), and the other, in both cases, corresponds to the frequency observed in the absence of product, 860 cm⁻¹. This indicates an equilibrium mixture of unbound and product-bound forms in solution following substrate oxidation.

The resonance Raman results show that BSO reductase and Me₂SO reductase differ with respect to the ability of the physiological product to effect reduction to the Mo(VI) state. Addition of a 20-fold excess of Me₂S to wild-type or redox-cycled Mo(VI) Me₂SO reductase results in reduction to the Mo(VI) state, as evidenced by resonance Raman (16), EXAFS (19), and x-ray absorption edge studies (6, 20). The Me₂S-reduced sample has an intense cherry red color due the emergence of two intense charge transfer bands at 480 and 540 nm (16, 32). These bands have been assigned to Mo(VI)-to-Me₂SO charge transfer on the basis of ¹⁸O/¹⁶O-labeling experiments, because laser excitation to these bands was found to enhance the Mo–O and O–S stretching modes of the bound Me₂SO, which occur at 497 and 862 cm⁻¹, respectively (16). BSO reductase has broad substrate specificity and can catalyze reduction of trimethyl-amine-N-oxide to TMA and Me₂SO to Me₂S. However, neither the physiological product biotin nor TMA or Me₂S was capable of reducing BSO reductase.

The addition of biotin, Me₂S, or TMA does, however, perturb the Mo=O stretching frequency, indicating a weak interaction between the product and the oxo group (Fig. 8). In the absence of a product molecule, the oxo group is depicted as being hydrogen bonded to Trp-90. This tryptophan corresponds to Trp-116 in the crystal structure of *R. capsulatus* Me₂SO reductase, which is hydrogen bonded via Nε1 to the long terminal oxo group (5, 6, 20). The other oxo group in this structure and the single oxo group in the *R. sphaeroides* Me₂SO reductase crystal structure are hydrogen bonded to the OH of Tyr-114 (2, 4–6). Although both of these residues are conserved in BSO reductase (Tyr-88 and Trp-90 (13)), only the tryptophan is conserved in all members of the Me₂SO reductase family (10). This observation and the crystallographic evidence showing that it is the oxo group hydrogen bonded to the tryptophan that interacts with Me₂S (6, 20) provide the rationale for preferring a hydrogen-bonding interaction with Trp-90 rather than Tyr-88. Hydrogen-bonding and interaction with product would both be expected to weaken the Mo–O bond, and whether or not the product-oxo interaction is in place of or in addition to the hydrogen-bonding interaction remains to be established. Hence, the observed Mo=O stretching frequencies indicate that interaction with Me₂S and TMA results in a Mo=O bond (ν(Mo=O) = 840 and 850 cm⁻¹, respectively) that is significantly weaker than in the as-prepared enzyme (ν(Mo=O) = 860 cm⁻¹). The strengthening of the Mo=O bond in the biotin-bound form of the enzyme (ν(Mo=O) = 890 cm⁻¹) is attributed to a much weaker product-oxo interaction, compared with Me₂S and TMA, coupled with loss of the hydrogen-bonding interaction. The substantial weakening of the Mo=O bond induced by interaction with Me₂S is presumably responsible for the observed ability to exchange the oxo group with solvent water in Me₂SO-reoxidized BSO reductase samples without redox cycling.
The resonance Raman results presented herein, in conjunction with the previously published resonance Raman (16) and crystallographic studies (2, 4–6 of Me₂SO reductase, have led to the proposed catalytic cycle of BSO reductase shown in Fig. 9. Ser-121, deduced to be a protein ligand to Mo on the basis of amino acid sequence alignment of BSO reductase (13) with other structurally characterized members of the Me₂SO reductase family (2, 4–6, 9–11), remains coordinated throughout the catalytic cycle. Our data do not as yet provide direct evidence for the coordinated water molecule in the reduced enzyme (C), and it is not possible to rule out a five-coordinate active site in the dithionite-reduced Mo(IV) form. Resonance Raman data for BSO reductase are currently available for the as-prepared oxidized enzyme (A), the product-associated oxidized enzyme (E), and the dithionite-reduced enzyme (C). In addition to demonstrating that both molybdenopterin dithiolenes are ligated in each of these three forms of the enzyme, the resonance Raman data can only be interpreted in terms of catalytic cycling between mono-oxo-Mo(VI) (A) and dioxo-Mo(V) (C) species. The ability to exchange the oxo group that was determined to be the site of attack by Me₂Si n towards R. capsulatus Me₂SO reductase (6, 20).

The resonance Raman results presented herein, in conjunction with 1H₂O/2H₂O exchange studies by1H2O/2H2O exchange studies of Me₂S-reduced BSO reductase (16) and BSO reductase have provided unique insights into their catalytic mechanisms and complement each other by characterizing the catalytic cycle (15). However, the overall oxygen atom transfer mechanism is clearly the same and is likely to be common to all members of this class of molybdenum oxotransferases.

In summary, the resonance Raman results for Me₂SO reductase (16) and BSO reductase have provided unique insights into their catalytic mechanisms and complement each other by facilitating characterization of different intermediate species. However, the overall oxygen atom transfer mechanism is clearly the same and is likely to be common to all members of this class of molybdenum oxotransferases.

**REFERENCES**
