Active Site Heterogeneity in Dimethyl Sulfoxide Reductase from *Rhodobacter capsulatus* Revealed by Raman Spectroscopy†

Alasdair F. Bell,‡ Xiang He,‡ Justin P. Ridge,§ Graeme R. Hanson,|| Alastair G. McEwan,§ and Peter J. Tonge*,‡

Department of Chemistry, SUNY at Stony Brook, Stony Brook, New York 11794-3400, and School of Molecular & Microbial Sciences and Centre for Magnetic Resonance, University of Queensland, St. Lucia, Queensland 4072, Australia

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ABSTRACT: Raman spectroscopy has been used to investigate the structure of the molybdenum cofactor in DMSO reductase from *Rhodobacter capsulatus*. Three oxidized forms of the enzyme, designated 'redox cycled', 'as prepared', and $DMSOR_{mod}D$, have been studied using 752 nm laser excitation. In addition, two reduced forms of DMSO reductase, prepared either anaerobically using DMS or using dithionite, have been characterized. The 'redox cycled' form has a single band in the Mo═O stretching region at 865 cm^{-1} consistent with other studies. This oxo ligand is found to be exchangeable directly with $DMS^{18}O$ or by redox cycling. Furthermore, deuteration experiments demonstrate that the oxo ligand in the oxidized enzyme has some hydroxo character, which is ascribed to a hydrogen bonding interaction with Trp 116. There is also evidence from the labeling studies for a modified dithiolene sulfur atom, which could be present as a sulfoxide. In addition to the 865 cm^{-1} band, an extra band at 818 cm^{-1} is observed in the Mo═O stretching region of the 'as prepared' enzyme which is not present in the 'redox cycled' enzyme. Based on the spectra of unlabeled and labeled DMS reduced enzyme, the band at 818 cm^{-1} is assigned to the S═O stretch of a coordinated DMSO molecule. The $DMSOR_{mod}D$ form, identified by its characteristic Raman spectrum, is also present in the 'as prepared' enzyme preparation but not after redox cycling. The complex mixture of forms identified in the 'as prepared' enzyme reveals a substantial degree of active site heterogeneity in DMSO reductase.

The mononuclear molybdoenzymes form a diverse but coherent group, characterized by the presence of one or two molecules of molybdopterin with dithiolene groups coordinated to a molybdenum center (*1*−*3*). Almost all molybdoenzymes catalyze a two-electron-transfer reaction that is linked to oxygen atom transfer to or from a water molecule. During catalysis, the molybdenum cycles between the Mo(VI) and Mo(IV) states. Dimethyl sulfoxide (DMSO) reductase from photosynthetic bacteria of the genus *Rhodobacter* is regarded as a key molybdenum enzyme for studies of structure−function relationships since it is one of only a very few examples of an enzyme which contains a molybdenum cofactor as its only prosthetic group. Thus, spectroscopic studies can proceed without interference from other chromophoric groups. The reaction catalyzed by this enzyme is shown in Figure 1. Our understanding of the structure of the molybdenum site in this enzyme has been transformed by X-ray crystallography (*4*−*8*), and this has been complemented by analysis using X-ray absorption spectroscopy (XAS) (*9*−*11*), resonance Raman spectroscopy (*12*−*14*), and EPR spectroscopy (*15*).

$$DMSO + 2H^+ + 2e^- \rightleftharpoons DMS + H_2O$$

FIGURE 1: Reaction catalyzed by DMSO reductase.

X-ray crystallographic studies have produced a surprisingly complex picture of the active site in DMSO reductase as three distinct, independent cofactor structures have been proposed (*4*−*6*). These structures differ from one another in two important respects: the number of oxo ligands and the nature of coordination of the dithiolene ligands. They do, however, agree in the overall structure of the protein and on the direct coordination of the cofactor to the protein via serine-147. A number of EXAFS studies have appeared which have helped to clarify some aspects of the structure of the molybdenum cofactor. In particular, the most recent EXAFS study on recombinant DMSO reductase from *Rhodobacter sphaeroides* proposed a six-coordinate cofactor structure which has a single oxo ligand and two equivalent dithiolene ligands and is also coordinated to serine-147 (*11*). A consensus has emerged that this oxo group (O2) is the one which hydrogen bonds to W116 since this is the oxo group which reacts with DMS to form a species with DMSO bound at the active site (*7*). This view is reinforced by the position of the single oxo in the revised crystal structure of DMSO reductase from *R. sphaeroides* (*8*). However, as a result, the oxo group that hydrogen bonds to Y114 (known as O1 in the structures described by Bailey and co-workers), and identified as the only oxo group in the first crystal

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* To whom correspondence should be addressed. Telephone: (631) 632 7907, Fax: (631) 632 7960, Email: Peter.Tonge@sunysb.edu.

‡ Department of Chemistry, SUNY at Stony Brook.

§ School of Molecular & Microbial Sciences, University of Queensland.

|| Centre for Magnetic Resonance, University of Queensland.

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structure of DMSO reductase (*4*), is no longer considered to be a component of the resting six-coordinate Mo(VI) form of *R. sphaeroides* DMSO reductase. In contrast, the seven-coordinate structure described by Bailey and co-workers (*6*) exhibits a trigonal arrangement of two oxo groups together with the serine side chain. This structure has been described as chemically unreasonable because of the small O=Mo=O bond angle and the crowding of groups in the Mo=O1, P-pterin S1 sulfur, and Oγ of S147 (*11*).

In this study, we describe the characterization of the molybdenum cofactor of DMSO reductase from *Rhodobacter capsulatus* using Raman spectroscopy. Resonance Raman studies on DMSO reductase from *R. sphaeroides* have previously been reported by Garton et al. (*13*). The resonance Raman study provided the first strong evidence for a single oxo group in DMSO reductase leading to a mono-oxo/des-oxo mechanism and identified two distinct, but fully coordinated, dithiolene ligands. This work has been further developed in a recent study on the molybdenum cofactor of the closely related enzyme biotin sulfoxide reductase (*16*). All recent work on *R. sphaeroides* DMSO reductase has used enzyme that has been 'redox cycled' by reduction with dithionite/methyl viologen followed by reoxidation. In the case of recombinant enzyme expressed in *E. coli*, redox cycling is essential to convert an inactive form of DMSO reductase, produced in the cytoplasm of the host, into an active species. In the current study, we have used enzyme prepared from the periplasm of *R. capsulatus*, which has enabled us to investigate by Raman spectroscopy material which is essentially the same as that used in spectroscopic and crystallographic studies (*6, 7, 10, 15*). Furthermore, we can compare directly the spectroscopic properties of the 'as prepared' form of DMSO reductase with 'redox cycled' enzyme.

EXPERIMENTAL PROCEDURES

Protein Preparation and Characterization. Dimethyl sulfoxide (DMSO), dimethyl sulfide (DMS), and sodium dithionite were obtained from Aldrich. Labeled water ($H_2{}^{18}O$) was obtained from Cambridge Isotope Laboratories and deuterium oxide (D_2O) (99.9% enriched) from Sigma. Labeled DMS^{18}O was prepared essentially as described previously (*17*). DMSO reductase was purified from *R. capsulatus* strain H123 essentially as described previously (*15, 18*) with some modifications. First, cells harvested after growth were washed in at least 2 × 1 L of 50 mM Tris-HCl (pH 8.0) to ensure removal of residual DMSO and DMS. Second, an additional purification step was introduced whereby pooled and concentrated fractions from the first column step were further purified using a Poros 50HQ anion exchange column (Perseptive Biosystems, USA). For the enzyme assays, "redox cycled" enzyme was generated under anaerobic conditions (<1 ppm O_2) as described previously (*11*), and DMSOR$_{mod}$D was generated as described previously (*19*). In both cases, excess reagents were then removed by passing the preparation through a PD10 size exclusion column (Amersham Pharmacia Biotech). DMSO reductase preparations were assayed in both the forward (DMSO reduction) and reverse (DMS oxidation) directions as described previously (*20, 21*). All assays were performed in triplicate.

Raman Spectroscopy. Raman spectra were acquired using an instrument that has been described in detail elsewhere (*22*). The Raman measurements were made by adding approximately 100 μL of enzyme to a specially designed anaerobic cell and collecting the data. Anaerobic conditions were only maintained for experiments involving dithionite and DMS reduction. After removal of the protein sample, the same volume of buffer (Tris-HCl, pH 8.0, 50 mM) was added to the same cell without making any changes to the optical alignment or to the cell position and the Raman spectrum measured. Difference Raman spectra were then calculated by performing a computer subtraction of the spectra of the enzyme in buffer minus the buffer alone. A typical experiment was completed in 8 min using 200 mW laser power with a protein concentration of around 50 μM determined by absorption spectroscopy using $A_{720} = 2$ mM^{-1} cm^{-1}. Since there is a possibility of laser damage to the sample, absorption spectra were taken before and after each Raman experiment, but no differences were observed. In addition, no changes were observed in the Raman spectrum of 'as prepared' enzyme even after exposure to 500 mW of laser power for 1 h, and a spectrum of 'as prepared' DMSO reductase recorded after only 30 s exposure to the laser, although noisy, revealed no differences to the spectrum reported here. The oxidized forms of the enzyme have weak fluorescence backgrounds; therefore, their difference spectra have been baseline-corrected. The difference spectra were wavenumber-calibrated against cyclohexanone and are accurate to ± 2 cm^{-1}. All spectral manipulations were carried out using Win-IR software, and data acquisition was performed using WinSpec (Princeton Instruments, Trenton, NJ). Under the conditions used for acquiring good quality difference Raman spectra, the resolution of our system is approximately 8 cm^{-1}.

RESULTS

General Raman Band Assignments for DMSO Reductase. Using 752 nm excitation, it is expected that the Raman spectrum of DMSO reductase will be dominated by bands due to normal modes involving the molybdenum cofactor. These specific bands will receive a preresonance or resonance intensity enhancement (depending on the oxidation state of the cofactor) as the exciting laser line is close in energy to electronic transitions associated with charge transfer between the dithiolene and oxo ligands and the molybdenum center. For the 'as prepared' oxidized form of the enzyme, absorption bands are observed at 723, 560, and 470 nm, while the anaerobically DMS reduced form is characterized by absorption bands at 546 and 478 nm. Thus, for the oxidized form of the enzyme, the laser excitation is within the wing of the 723 nm absorption band, and structural features associated with this transition will dominate the Raman spectrum. However, for DMS reduced enzyme, the 546 and 478 nm transitions will be important in determining which normal modes are intensity-enhanced. Some of the weaker bands in the Raman spectrum, that are not selectively boosted in intensity, are attributable to the protein and can be identified from their characteristic band positions. In particular, bands at around 1650 cm^{-1} and in the range 1350−1220 cm^{-1} are assigned to amide I and amide III modes, respectively. In addition, a prominent band near 1450 cm^{-1} is assigned to CH_2 modes of the protein side chains.

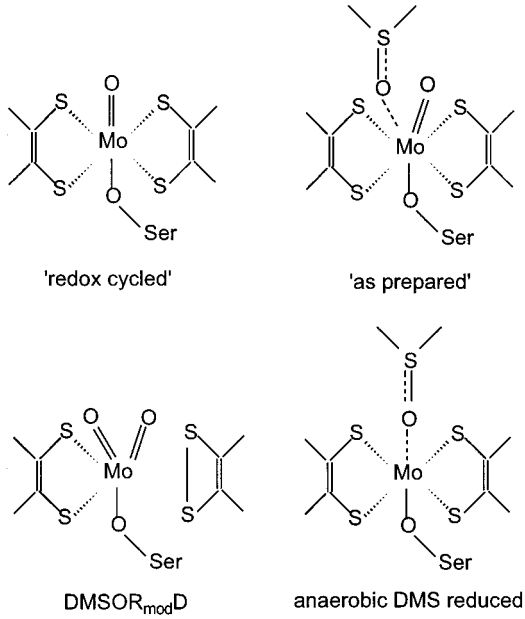

FIGURE 2: Proposed cofactor structures for 'redox cycled', 'as prepared', DMSOR$_{mod}$D, and anaerobically DMS reduced DMSO reductase from *R. capsulatus*.

Based on earlier resonance Raman studies, the normal modes assigned to the cofactor can be conveniently subdivided into four distinct spectral regions: the Mo−S stretching modes of the dithiolene ligands (300−400 cm^{-1}), the Mo═O stretching modes of oxo ligands (800−900 cm^{-1}), the coupled C−C and C−S stretching modes of the dithiolene ligands (1000−1180 cm^{-1}), and the C═C stretching modes of the dithiolene ligands (1500−1600 cm^{-1}). In the current study, we will concentrate mainly on the Mo═O, Mo−S, and C═C stretching regions. Bands attributable to these modes are easily identifiable by comparison with earlier resonance Raman studies (*12*−*14*) and appear in spectral regions that do not contain strong contributions from the protein. Using these assignments, we will compare and contrast the 'redox cycled' and 'as prepared' enzyme, explore the effects of DMS and dithionite reduction, and identify bands due to exchangeable oxygen and hydrogen atoms using isotopic exchange. As a guide to the following discussion, the structures that we propose for the different forms of the enzyme that we have obtained Raman spectra for are shown in Figure 2.

Raman Spectroscopy of 'Redox Cycled' Enzyme. The Raman spectrum of 'redox cycled' DMSO reductase from *R. capsulatus* between 600 and 1800 cm^{-1} is shown in Figure 3A. This spectrum is similar to that presented by Garton et al. in an earlier resonance Raman study on 'redox cycled' DMSO reductase from *R. sphaeroides* (*13*). However, there are some changes in the relative intensities, the resolution, and the exact positions of bands that can be ascribed to differences in the excitation wavelength and experimental conditions used in the current study (see Experimental Procedures). In an attempt to relate structure and reactivity, we have measured the catalytic activities in forward (methyl viologen) and reverse (DMS:DCPIP) assays for the three oxidized forms of the enzyme, and the results are shown in Table 1. The 'redox cycled' enzyme has the highest catalytic activity of the forms of the enzyme examined, but we only observe small differences between the various enzyme forms

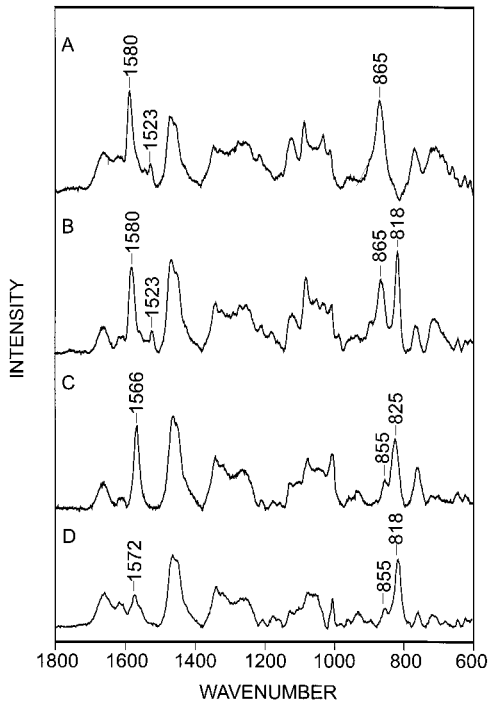

FIGURE 3: Raman spectra of DMSO reductase from *R. capsulatus* in Tris buffer (pH 8.0, 50 mM) between 1800 and 600 cm^{-1}. (A) 'Redox cycled' with 10 mM dithionite and 100 mM unlabeled DMSO. (B) 'As prepared' enzyme. (C) DMSOR$_{mod}$D form generated by aerobic treatment of 'redox cycled' enzyme with 100 mM DMS for 24 h. (D) 'As prepared' enzyme anaerobically DMS reduced. Excess reductants were removed from DMSOR$_{mod}$D by three 10-fold dilution/reconcentration steps. Enzyme concentrations were between 50 and 100 μM. Spectra were acquired for 8 min using 200 mW of 752 nm excitation.

Table 1: Catalytic Activity in Forward (MV) and Reverse (DMS:DCPIP) Assays of 'Redox Cycled', 'As Prepared', and DMSOR$_{mod}$D Forms of DMSO Reductase from *R. capsulatus*

DMSOR form	assay	k_{cat} (s^{-1})[a]
'as prepared'	MV	26.98 ± 0.1
	DMS:DCPIP	2.23 ± 0.3
'redox cycled'	MV	34.61 ± 2.0
	DMS:DCPIP	2.97 ± 0.1
DMSOR$_{mod}$D	MV	31.91 ± 1.5
	DMS:DCPIP	1.17 ± 0.0[b]

[a] k_{cat} is an average of 3 measurements. [b] The error for the DMS:DCPIP assay on DMSOR$_{mod}$D is in fact 0.02 but has been rounded to 2 significant figures.

in both assays. We have also recorded absorption spectra for the different forms of the enzyme we have studied, and these are shown in Figure 4. The absorption spectrum of 'redox cycled' enzyme closely matches those previously reported.

In the Mo═O stretching region (800−900 cm^{-1}), the 'redox cycled' enzyme exhibits a single band at 865 cm^{-1}, consistent with a single monooxo form of the enzyme being present (*23*). A powerful method for identifying bands associated with Mo═O stretching modes is to introduce ^{18}O isotopic labels (*14*, *23*, *24*, *30*). For a monooxo structure, an isolated Mo═O stretching band is expected to shift by slightly more than 40 cm^{-1}. In contrast, in a dioxo structure, with two coupled stretching modes, both bands are expected to be sensitive to labeling even if only one of the oxo ligands has ^{18}O incorporated. We have found that simply exchanging

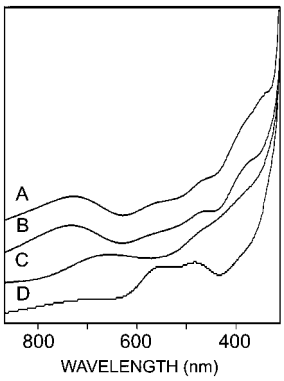

FIGURE 4: Absorption spectra for (A) 'redox cycled' enzyme. (B) 'As prepared' enzyme. (C) $DMSOR_{mod}D$ generated from 'redox cycled' enzyme. (D) 'As prepared' enzyme anaerobically DMS reduced.

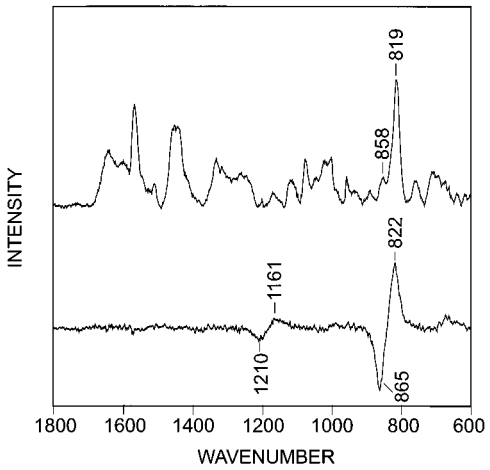

FIGURE 5: (A) Raman spectrum of 'redox cycled' DMSO reductase after direct exchange with $DMS^{18}O$. (B) Isotope-edited spectrum generated by subtraction of unlabeled 'redox cycled' enzyme from ^{18}O-labeled enzyme. Excess $DMS^{18}O$ was removed by three 10-fold dilution/reconcentration steps. Enzyme concentration was 50 μM. Spectra were acquired for 8 min using 200 mW of 752 nm excitation.

the enzyme into ^{18}O-labeled buffer, without reducing and reoxidizing the enzyme, does not cause any significant change in the Raman spectrum even after 2 weeks at 4 °C (data not shown). However, 25 equiv of ^{18}O-labeled DMSO, without any reductant present, can be used to incorporate labeled oxygen *directly* into the molybdenum cofactor as shown in Figure 5. This labeling experiment on 'redox cycled' enzyme results in the shift of a single band in the Mo═O stretching region at 865 cm^{-1} to 819 cm^{-1} (−46 cm^{-1}). This demonstrates that there is a single exchangeable oxo ligand directly bonded to the metal center in the 'redox cycled' enzyme. Almost identical isotope shifts are observed for enzyme that is subjected to redox cycling in ^{18}O-labeled buffer or to redox cycling in unlabeled buffer with either DMS or dithionite as a reductant and $DMS^{18}O$ used as a reoxidant (data not shown). Control experiments revealed no change in the Raman bands attributable to the cofactor upon addition of an excess of unlabeled DMSO to 'redox cycled' enzyme.

Further information on the cofactor structure is available from the Mo−S (300−400 cm^{-1}; Figure 6) and C═C (1500−1600 cm^{-1}; Figure 3) stretching regions of the dithiolene ligands. An early resonance Raman study on

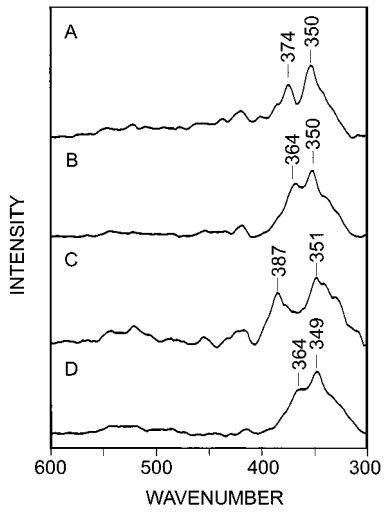

FIGURE 6: Raman spectra of DMSO reductase in Tris buffer (pH 8.0, 50 mM) between 600 and 300 cm^{-1}. (A) 'Redox cycled' enzyme. (B) 'As prepared' enzyme. (C) $DMSOR_{mod}D$ enzyme. (D) Anaerobically DMS reduced enzyme. For experimental conditions, see caption for Figure 3.

DMSO reductase revealed that bands attributed to Mo−S stretching modes were sensitive to the oxidation state (*12*). In particular, for oxidized enzyme an intense band was observed at 350 cm^{-1} with two weaker bands appearing at 371 and 379 cm^{-1}. In the current study, the 'redox cycled' enzyme exhibits an intense signal at 350 cm^{-1} and a weaker band at 374 cm^{-1} (Figure 6A). The lower resolution of the spectra in the current study means that the two bands at 371 and 379 cm^{-1} are not resolved, presumably as a consequence of the higher temperature at which the spectra have been recorded. It is also informative to examine the C═C stretching region where the bands report on the nature of the coordination of the dithiolene ligands (*12, 13*). In this region, a strong band at 1580 cm^{-1} and a weaker band 1523 cm^{-1} appear in the spectrum of 'redox cycled' enzyme. The fact that two C═C stretching bands are observed has been attributed to the presence of two distinct, but fully coordinated, dithiolene ligands (*13*).

Raman Spectroscopy of DMS Reduced Forms of DMSO Reductase. It is known that formation of the DMS reduced form of DMSO reductase is fully reversible after brief exposure to DMS in the presence of oxygen or for longer periods under anaerobic conditions. However, extended aerobic exposure to DMS generates a new form of the enzyme that has been designated $DMSOR_{mod}D$ (*19, 25*). The $DMSOR_{mod}D$ form has been examined in detail and found to have a distinctive absorption spectrum containing a characteristic band at 660 nm. This oxidized form has been proposed to be a contaminant in some preparations of the enzyme. We have examined the Raman spectra of 'redox cycled' enzyme that has been converted into the $DMSOR_{mod}D$ form as well as enzyme that has been DMS reduced under anaerobic conditions, and these data are shown in Figure 3C,D. To identify which particular enzyme forms are present in each case, the absorption spectra of the samples used in the Raman experiments have been recorded and are shown in Figure 4. The $DMSOR_{mod}D$ form shows close to normal activity in the forward assay but about one-third of the activity compared to 'redox cycled' enzyme in the reverse assay (Table 1).

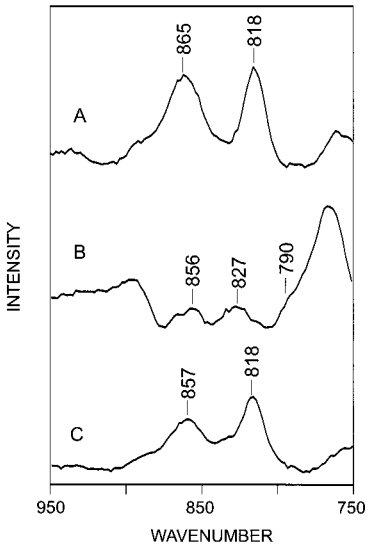

FIGURE 7: Raman spectra of DMSO reductase in the Mo═O stretching region of (A) 'As prepared' enzyme in Tris buffer (pH 8.0, 50 mM). (B) 'Redox cycled' with DMS^{18}O to incorporate labeled oxygen followed by DMS reduction. (C) 'Redox cycled' enzyme exchanged into deuterated Tris buffer (pH 8.0, 50 mM).

The DMSOR$_{mod}$D Raman spectrum (Figure 3C), generated from 'redox cycled' enzyme, exhibits two bands in the Mo═O stretching region at 855 and 825 cm^{-1}. An almost identical spectrum is observed for the DMSOR$_{mod}$D enzyme produced from 'as prepared' enzyme. However, 'as prepared' enzyme that has been reduced with DMS under anaerobic conditions produces a distinct Raman spectrum containing a single intense band at 818 cm^{-1} and a much weaker band at 855 cm^{-1} (Figure 3D). This difference between the Raman spectra provides a means to discriminate between the DMSOR$_{mod}$D and anaerobically DMS reduced forms of the enzyme. In addition, a Raman study on model dioxomolybdenum compounds revealed that the symmetric and antisymmetric combinations of the two Mo═O stretches produce two bands in the Mo═O stretching region (*30*). These two bands are separated on average by around 30 cm^{-1}, providing support for the idea that the DMSOR$_{mod}$D is a dioxo form. Unfortunately, we have not been able to introduce isotope labels into the DMSOR$_{mod}$D form to confirm this structure except by redox cycling with ^{18}O-labeled DMSO. These conditions regenerate the 'redox cycled' form of the enzyme, and the same isotope shift is observed as reported above.

The crystal structure of the DMS reduced enzyme from *R. capsulatus* reveals a DMSO molecule coordinated to the Mo atom through an oxygen atom in the position of O2 (*7*). On this basis, it seems likely that the 818 cm^{-1} band characteristic for anaerobic DMS reduced enzyme can be assigned to the S═O stretch of a DMSO molecule coordinated to the molybdenum center through the oxygen atom. To confirm this assignment, we have incorporated ^{18}O into the cofactor by redox cycling enzyme with dithionite and labeled DMS^{18}O and then recorded the Raman spectrum after DMS reduction (Figure 7B). This procedure is expected to produce a reduced form of the cofactor with a ^{18}O-labeled DMSO molecule coordinated to the metal center (*13*). The labeled DMS reduced spectrum has lost intensity at 818 cm^{-1}, and a new band has appeared at around 790 cm^{-1}. This new band appears as a shoulder on the 765 cm^{-1} band which gains intensity relative to other bands in the DMS reduced enzyme. The shift of less than 30 cm^{-1} is consistent with a S═O stretching mode (the expected value would be >40 cm^{-1} for a pure Mo═O stretch). Furthermore, the low frequency of this mode, compared to 1050 cm^{-1} for DMSO in water, reveals that the S═O has lost a substantial amount of its double bond character on coordination with the Mo center. The fact that we can observe this band with 752 nm excitation indicates that there is charge-transfer transition between the DMSO and the molybdenum center. We note that two weak bands at 856 and 827 cm^{-1} remain after the labeling experiment. The positions of these bands indicate that they could be associated with a small population of the DMSOR$_{mod}$D form that has not been reduced.

Both the DMSOR$_{mod}$D and anaerobically DMS reduced forms have characteristic bands in the Mo−S stretching region which can help in identifying which forms are present. In particular, for DMSOR$_{mod}$D, two bands are observed at 351 and 387 cm^{-1} whereas for anaerobic DMS reduced enzyme two bands appear at 349 and 364 cm^{-1} (Figure 6C,D). In the C═C stretching region, DMS treatment of 'redox cycled' enzyme to produce the DMSOR$_{mod}$D form results in the disappearance of the weak 1523 cm^{-1} band and a shift of the intense band at 1580 cm^{-1} to 1566 cm^{-1}. In contrast, for anaerobically DMS reduced enzyme, the band at 1580 cm^{-1} is shifted to 1572 cm^{-1} and has become much broader while the 1523 cm^{-1} band has disappeared. In principle, the changes in the C═C stretching modes for DMSOR$_{mod}$D could indicate that only one dithiolene ligand remains coordinated or that the two dithiolene ligands have become equivalent following DMS treatment and air reoxidation.

Raman Spectroscopy of 'As Prepared' Enzyme. The 'as prepared' enzyme is produced by conventional chromatography from a periplasmic fraction prepared from *R. capsulatus* grown phototrophically in the presence of DMSO (*18*). The catalytic activity is only slightly reduced relative to enzyme that has been 'redox cycled' as determined by the forward and reverse assays (Table 1). Furthermore, the absorption spectrum is similar to those reported elsewhere and does not seem to indicate that a large population of DMSOR$_{mod}$D is present.

The Mo═O stretching region of the 'as prepared' enzyme contains two bands at 865 and 818 cm^{-1} (Figure 3B). By comparison with the spectra discussed above, we assign the bands at 865 and 818 cm^{-1} to an oxo ligand and a coordinated DMSO molecule, respectively. A barely perceptible shoulder at 855 cm^{-1} is also observed which may indicate that there is some of the DMSOR$_{mod}$D form present in this preparation. Direct isotope labeling with DMS^{18}O of the 'as prepared' enzyme produces only a shift in the 865 cm^{-1} band to 822 cm^{-1}, almost identical to that observed for 'redox cycled' enzyme and consistent with a single oxo ligand. The 818 cm^{-1} band corresponds to the band at the same wavenumber in the anaerobically DMS reduced enzyme and is assigned to a coordinated DMSO molecule. These data indicate that there is some structural similarity between the 'as prepared' and anaerobically DMS reduced enzyme but that the 'as prepared' enzyme is distinct from the DMSOR$_{mod}$D form.

Further support for this interpretation is found in the Mo−S (300−400 cm^{-1}) stretching region (Figure 6). For

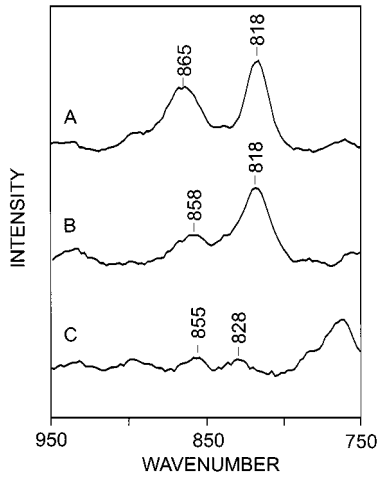

FIGURE 8: Raman spectra of DMSO reductase in the Mo═O stretching region after dithionite reduction. (A) 'As prepared' enzyme prior to reduction. (B) 20 equiv of dithionite added. (C) 50 equiv of dithionite added. Enzyme concentration was 50 μM. Spectra were acquired for 8 min using 200 mW of 752 nm excitation.

the $DMSOR_{mod}D$ form of the enzyme, characteristic bands are observed at 351 and 387 cm^{-1}, and for anaerobically DMS reduced enzyme, bands appear at 349 and 364 cm^{-1}. However, the 'as prepared' enzyme consists of a doublet of bands of nearly equal intensity at 350 and 364 cm^{-1} (Figure 6). The band positions in the 'as prepared' enzyme match most closely those observed in the anaerobically DMS reduced enzyme.

To help understand how redox cycling produces a simpler Raman spectrum than the 'as prepared' enzyme, we have examined the effect of dithionite reduction as shown in Figure 8. Our results indicate that there is a concentration dependence for dithionite reduction. At lower concentration (20 equiv), the 865 cm^{-1} band is specifically removed (Figure 8B). However, at higher dithionite concentration (50 equiv), both the 865 and 818 cm^{-1} bands are lost, revealing two weak bands at 855 and 828 cm^{-1} that can be assigned to the $DMSOR_{mod}D$ form of the enzyme (Figure 8C). Subsequent reoxidation with an excess of DMSO produces only the monooxo oxidized ('redox cycled') form of the enzyme as judged by the presence of a single Raman band at 865 cm^{-1}. For 'redox cycled' enzyme, addition of dithionite (20 equiv) removes the 865 cm^{-1} band. These data indicate that the 865 and 818 cm^{-1} bands are attributable either to a single species containing two ligands, one of which is easier to reduce than the other, or to two distinct species with different susceptibilities to reduction. Although the relative populations of the various forms of the enzyme are uncertain due to their different levels of resonant enhancement, the $DMSOR_{mod}D$ form is shown to have only a weak influence on the spectra of 'redox cycled' and 'as prepared' enzyme.

Evidence for the Hydroxo Character of the Oxo Ligand. In an attempt to discover whether any hydroxo (OH) or aquo (H_2O) ligands are present in the oxidized or reduced forms, we have exchanged 'redox cycled' and 'as prepared' enzyme into deuterated buffer. Upon deuteration, modes associated with Mo−O−H bending motions would be expected to exhibit large wavenumber shifts (>100 cm^{-1}) if either OH or H_2O ligands are coordinated to the metal center (*26*, *27*). The Raman spectrum of 'redox cycled' enzyme exchanged into deuterated buffer is shown in Figure 7C. Since there are no changes observed on the order of hundreds of wavenumbers, we find no evidence for bending modes of either hydroxo or aquo ligands. This could be because such bands are not resonantly enhanced using 752 nm excitation. However, the band that appears at 865 cm^{-1} in H_2O, assigned to the exchangeable Mo═O stretch, shifts to 858 cm^{-1} (−7 cm^{-1}) in deuterated buffer. It is known that the Mo═O stretching band of a Mo−O−H group will shift by between about 10 and 25 cm^{-1} on deuteration (*27*). This result indicates that in the oxidized enzyme the oxo ligand has at least some partial hydroxo character most likely due to a hydrogen bonding interaction involving the oxo ligand. No change is observed in the Raman spectrum of enzyme that has been exchanged into deuterated buffer and then DMS-reduced (data not shown).

Evidence for a Metallosulfoxide. Examination of Figure 5 reveals that the shift of the 865 cm^{-1} band assigned to a Mo═O stretching mode is not the only change observed on ^{18}O-labeling. A second weak band is observed at 1210 cm^{-1} in the oxidized enzyme that shifts to 1161 cm^{-1} (−49 cm^{-1}) upon addition of labeled $DMS^{18}O$. Unfortunately, the bands in this spectral region are rather weak, and those due to the cofactor are masked to some extent by protein bands. However, the isotope-edited spectra reveal a clear difference in this region in both 'redox cycled' (Figure 5) and 'as prepared' enzyme (data not shown). This band has previously been assigned to a combination mode of the Mo═O (862 cm^{-1}) and Mo−S stretching (350 cm^{-1}) fundamentals (*13*). On the basis of a simple diatomic oscillator model, the observed wavenumber shift (−49 cm^{-1}) is consistent with the labeling of an S═O fundamental (calculated to be −46 cm^{-1} for a pure diatomic at 1210 cm^{-1}). The S═O stretch of sulfoxides in solution typically appears around 1050 cm^{-1} and is known to be relatively insensitive to the effects of either conjugation or hydrogen bonding (*28*). However, a number of complexes have been reported where a sulfoxide is coordinated to a metal center through the sulfur atom, leading to a large increase in the frequency of the S═O stretch (*26*). These observations lead to the interesting possibility that one of the dithiolene ligands is oxidized to form an S═O bond where the sulfur atom is coordinated to the molybdenum center.

DISCUSSION

From the available Raman data, with particular emphasis on the isotope labeling experiments, we propose the structures for the 'redox cycled', 'as prepared', and $DMSOR_{mod}D$ oxidized forms as well as the anaerobically DMS reduced form of DMSO reductase shown in Figure 2. In the Mo═O stretching region, bands are found that are characteristic for the different forms of the enzyme that we have generated, and these are summarized in Table 2. The 865 cm^{-1} band is assigned to a Mo═O stretching mode and is characteristic for an oxo ligand. Furthermore, on the basis of the observed wavenumber shift (−46 cm^{-1}) and on the lack of other changes in this spectral region on labeling of the oxo ligand, the Raman data on the 'redox cycled' enzyme are only consistent with a monooxo structure as previously reported (*13*). This oxo group is almost certainly equivalent to O2 in the structures described by Bailey and co-workers (*6*, *7*). In the 'redox cycled' enzyme spectrum, this is the only band

Table 2: Raman Band Positions in the Mo═O Stretching Region for Oxidized and Reduced Forms of DMSO Reductase from *R. capsulatus*

experimental conditions	Raman band position in wavenumbers	
'redox cycled'	865	–
'as prepared'	865	818
'redox cycled'/$DMSOR_{mod}D$	855	825
'as prepared'/$DMSOR_{mod}D$	855	824
'as prepared'/dithionite (20 equiv)	858 (weak)	818
'as prepared'/dithionite (50 equiv)	855 (weak)	828 (weak)

observed in the Mo═O stretching region even when a large excess of DMSO (100 mM) is added.

For the 'as prepared' enzyme, we observe two Raman bands in the Mo═O stretching region. From a comparison with the spectra of the 'redox cycled' and the anaerobically DMS reduced forms, these two bands at 865 and 818 cm^{-1} can be assigned to a single oxo ligand and a coordinated DMSO molecule, respectively. The 865 cm^{-1} band in the 'as prepared' enzyme exhibits almost the same isotope shift (-43 cm^{-1}) as for 'redox cycled' enzyme on exchange with $DMS^{18}O$, identifying it as O2. The 818 cm^{-1} band, characteristic for the anaerobically DMS reduced form of the enzyme, is assigned to the S═O stretch of a DMSO molecule coordinated to the metal center through its oxygen atom. This assignment is supported by the 28 cm^{-1} shift to lower wavenumber observed for anaerobic DMS reduced enzyme that has been labeled with ^{18}O. This stretching frequency is about 40−45 cm^{-1} lower than observed for DMS reduced enzyme from *R. sphaeroides* (*13*) and indicates a weaker S═O bond for the bound DMSO molecule in *R. capsulatus* presumably due to stronger coordination with the molybdenum center.

In principle, the oxo and DMSO ligands in 'as prepared' enzyme could both be present in a single species, or there could be a mixture of two different forms, one of which has only an oxo ligand and the other which has only a coordinated DMSO molecule. Unfortunately, the Raman data cannot distinguish between these two possibilities. Furthermore, there is no change in the ratio of intensities of the two bands at 865 and 818 cm^{-1} between pH 5 and 8 or as a function of concentration between 10 and 300 μM (unpublished observations). Johnson and co-workers in a resonance Raman study of DMSO reductase from *R. sphaeroides* noted that 'redox cycled' enzyme produces a sharper Raman spectrum than 'as prepared' (*13*). This is to be expected since it is now known that redox cycling will convert $DMSOR_{mod}D$ and other damaged forms of the enzyme into the 'redox cycled' form (*19, 25*). Therefore, this observation does not necessarily imply that two species are required to produce the two bands found in 'as prepared' enzyme. However, they also proposed a product (DMS)-bound intermediate for DMSO reductase from *R. sphaeroides* and observed a product-bound monooxo form for biotin sulfoxide (*16*). The present study demonstrates that the stable intermediate in DMSO reductase from *R. capsulatus* is a substrate (DMSO)-bound form. Regardless of whether a single species or two species generate the 865 and 818 cm^{-1} bands in 'as prepared' enzyme, it is important to note that this observation provides a clear difference between enzyme that has been redox cycled and that which has not. However, the structural differences identified between 'redox cycled' and 'as-prepared' enzymes do not result in significant differences in the enzyme assays using these two forms of the enzyme. The lack of observable difference in reactivity is most likely a consequence of the strong oxidizing or reducing conditions that are used or the nonphysiological routes of electron transfer involving redox dyes. In this context, it would be interesting to compare the activity of the different forms of DMSO reductase with its physiological electron donor, the cytochrome DorC (*29*).

In the Mo═O stretching region, we observe some broadening of the Raman band at 865 cm^{-1} in the 'as prepared' enzyme relative to the same band in the spectrum of 'redox cycled' enzyme. This may indicate that $DMSOR_{mod}D$, which is characterized by Raman bands at 855 and 825 cm^{-1} and can be converted into the monooxo form by redox cycling, is a contaminant in the 'as prepared' enzyme. Due to the different characteristic absorption spectra of 'as prepared' and $DMSOR_{mod}D$ enzyme forms, and hence the degree of resonant enhancement with 752 nm excitation, it is difficult to quantify the populations of the two forms. However, the characteristic band positions for $DMSOR_{mod}D$ are distinct from those observed in either 'as prepared' or 'redox cycled' enzyme. In addition, the $DMSOR_{mod}D$ form is characterized by some interesting changes in the C═C stretching region (1500−1600 cm^{-1}) where bands sensitive to the coordination of the dithiolene ligands are found. In 'redox cycled' and 'as prepared' enzyme, two bands at 1580 and 1523 cm^{-1} are observed, but only a single band at 1566 cm^{-1} is found for $DMSOR_{mod}D$. Earlier resonance Raman studies ascribed the presence of two bands to two distinct coordinated dithiolene ligands. In this case, the changes observed in the C═C stretching bands may indicate that for $DMSOR_{mod}D$ one of the dithiolene ligands has dissociated. Alternatively, the experimental data could indicate that the two nonequivalent dithiolene ligands have become equivalent in the $DMSOR_{mod}D$ form. The latter possibility would be more consistent with a recent study by Bray et al. on oxygen-damaged forms of DMSO reductase, which combined a detailed analysis of absorption spectra with new crystallographic data. This study uncovered forms of the enzyme in which one of the dithiolene ligands had dissociated (*25*). These forms are thought to be a significant contaminant in some preparations of the enzyme and are characterized by absorption spectra which only contain bands below 450 nm. These species do not strongly impact on the current study since they will not be resonantly enhanced. Recently, a revised crystal structure (at 1.3 Å resolution) of DMSO reductase from *R. sphaeroides* was presented. This study concluded that the cofactor was present as a mixture of two populations, one of which was monooxo, hexacoordinate and the other dioxo, pentacoordinate (*8*). In these two forms, the molybdenum atom shifts by about 1.6 Å within the active site, and it is thought that the molybdenum atom in the minor population could have been mistaken for a second oxo ligand in earlier studies. Comparison of the crystallographic data with our Raman data would seem to indicate that the hexacoordinate form present in the crystal corresponds to the 'redox cycled' enzyme in the current study. The origin of the pentacoordinate form is more difficult to reconcile with the Raman data but could correspond to the $DMSOR_{mod}D$

form or perhaps to the dithiolene-dissociated forms identified by Bray et al. (*25*).

DMSO reductase catalyzes an oxo transfer reaction (Figure 1), and our results highlight how easily the oxo ligand can be replaced in the oxidized enzyme. We have found that this particular oxygen will exchange directly with labeled $DMS^{18}O$ without any reduction step. In contrast, labeled solvent water requires a redox cycle to become incorporated. The position of the Mo═O stretching band (865 cm^{-1}) is around 40−50 cm^{-1} lower than generally observed for the Mo═O stretch of model monooxo molybdenum compounds containing dithiolene ligands (*14, 24, 30*). This lowering of the stretching frequency is indicative of a weakening of the Mo═O bond and is consistent with the lability of this ligand. A relationship between Mo−O bond length and vibrational frequency of the Mo−O stretch has been developed for molybdenum oxides (*31*). Applying this equation to the shift of approximately 50 cm^{-1} for the Mo═O stretch in DMSO reductase relative to model compounds can be used to estimate a bond length difference on the order of 0.027 Å (1.728 Å for model to 1.755 Å for enzyme). Furthermore, on the basis of the deuteration results described above, it appears that the oxo ligand actually has some weak hydroxo character as reflected by the 7 cm^{-1} shift to lower wavenumber of the Mo═O stretching band for enzyme exchanged into deuterated buffer. The most likely explanation for this deuteration shift is a weakening of the Mo═O bond as a result of hydrogen bonding interactions between O2 and Trp-116 as identified in the crystal structure of 'as prepared' DMSO reductase from *R. capsulatus* (*6*). This intrepretation is consistent with the suggestion of Garner and co-workers (*10*) that the labile oxo ligand should be considered a 'light' oxo group with a longer Mo−O bond distance than observed for a typical oxo ligand.

The isotope labeling studies also provide evidence for a second exchangeable oxygen atom. Specifically, a 49 cm^{-1} shift to lower wavenumber of the band at 1210 cm^{-1} is observed (Figure 5). It has been suggested previously that this band may be assigned to a combination mode involving the Mo═O and Mo−S fundamentals since it exhibits approximately the same shift as observed in the Mo═O fundamental upon labeling (*13*). We provide an alternative interpretation in which this mode is assigned to an S═O stretching fundamental (expected to shift by 46 cm^{-1}). The unusually high frequency of this mode indicates that the sulfur atom is coordinated to the molybdenum center (*26*). This opens up the interesting possibility that there is an outer-sphere oxygen attached to one of the dithiolene ligands (*32*). A recent crystal structure described by Schindelin and co-workers (*8*) identified some unexplained regions of electron density, particularly around one of the dithiolene sulfurs, that could perhaps be associated with some modification to the coordinated sulfur atom. This could also provide an explanation for the observation of two distinct C═C stretching modes at 1580 and 1523 cm^{-1} in 'redox cycled' and 'as prepared' enzyme. If only one dithiolene ligand is oxidized, then the S═O bond would be in conjugation with the C═C and could lower the frequency of the C═C stretch. Thus, the 1523 cm^{-1} band would be associated with a dithiolene ligand with an S═O group whereas the band at 1580 cm^{-1} would be from a dithiolene ligand lacking this moiety. After DMS (or dithionite) reduction and in the $DMSOR_{mod}D$ form, only a single band is observed in the C═C stretching region, consistent with either two equivalent dithiolene ligands or loss of one of the dithiolene ligands. It may be that the reduced and $DMSOR_{mod}D$ forms lack this modification to the sulfur atom of one of the dithiolene ligands.

We propose that the S═O group is located at the P-pterin S1 sulfur. It is becoming increasingly clear that sulfenic acids and related groups have key roles in enzyme catalysis and redox regulation (*33*). The presence of an oxygen atom at S1P could explain the plasticity of the active site of DMSO reductase, and it could also have a key role in oxygen atom transfer during the catalytic cycle.

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